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EFFECT OF ACETATE, CITRATE, AND DIVALENT METAL IONS ON UTILIZATION OF SODIUM CASEINATE BY LACTIC STREPTOCOCCI[1]

I. J. McDONALD

Abstract

Growth of *caseinate utilizing* lactic streptococci in media with sodium caseinate as the nitrogen source was retarded by sodium acetate (40 mg./ml.) and by sodium citrate (10 mg./ml.). The inhibition caused by citrate was relieved by calcium or magnesium while that caused by acetate was relieved by calcium. In media with acid hydrolyzed casein as the nitrogen source, growth was inhibited in the presence of sodium citrate (10 mg./ml.) or disodium ethylene diamine tetraacetate (0.004 mg./ml.) but this inhibition was overcome by the addition of either calcium or magnesium. It appears, therefore, that these divalent ions were not required merely for activation of proteases produced by the organisms.

Introduction

Acetate and citrate have been shown to affect the growth of lactic streptococci in an amino acid medium (5). At acetate concentrations of 0.1 to 0.3%, growth was stimulated, whereas above 0.3% growth was inhibited. In the presence of 0.1 or 1.0% citrate, growth of *Streptococcus lactis* strains was stimulated whereas growth of *Streptococcus cremoris* strains was inhibited. Other workers (9, 8) have shown that *Streptococcus faecalis* and species of lactobacilli require divalent metal ions for growth in the presence of acetate or citrate. Three strains of lactic streptococci have been shown to grow with unhydrolyzed sodium caseinate as the nitrogen source in a chemically defined medium (7). Since this medium contained 2% sodium acetate, the following study was undertaken to determine the effect of some divalent metals on the growth of lactic streptococci in media containing acetate or citrate and unhydrolyzed sodium caseinate as the nitrogen source.

Materials and Methods

Streptococcus lactis Strain 829 and *Streptococcus cremoris* Strains H22-10 and H22-12 (7) were maintained by weekly transfer in reconstituted skim milk (Difco) containing 0.5% glucose and 0.5% yeast extract (Difco).

[1]Manuscript received November 25, 1956.
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These cultures grew in a chemically defined medium with unhydrolyzed sodium caseinate as the nitrogen source and have been designated as *caseinate utilizing* strains.

The basal medium described previously (Medium I (6)) was modified by the omission of cysteine hydrochloride and sodium acetate. Analytical reagent disodium ethylene diamine tetraacetate (EDTA), and the reagent grade chemicals: sodium acetate trihydrate, sodium citrate dihydrate, magnesium sulphate (anhydrous), calcium chloride (anhydrous), lithium chloride (anhydrous), potassium chloride (crystal), barium chloride dihydrate, and strontium chloride hexahydrate were added as required. The amounts of metallic ions indicated in tables and figures were in addition to those already present in the basal medium. Two per cent solutions of sodium caseinate (Nutritional Biochemicals Corp.) were prepared and passed through a Seitz filter (6). Vitamin free casein (Nutritional Biochemicals Corp.) was refluxed with constant boiling hydrochloric acid for 10 hours and the hydrolyzate treated at pH 3.5 and 6.0 with activated carbon (13).

The medium, without sodium caseinate, was adjusted to pH 6.6 and autoclaved for 15 minutes at 15 lb. pressure. Solutions of sodium caseinate, sterilized by filtration, were added to the cooled medium to give a final concentration of 2.0 mg. per ml. When they were used, acid hydrolyzed casein, EDTA, acetate, citrate, and salts of magnesium, calcium, potassium, lithium, barium, and strontium were sterilized with the other components of the medium.

Cells used as inoculum were centrifuged from 18- to 24-hour cultures in tryptone–tryptose broth (Medium II (6)), washed three times, and diluted to their original volume with sterile 0.9% saline. An inoculum of 0.1 ml. per 10 ml. of medium was used. After incubation at 30° C., growth was measured turbidimetrically in media with acid hydrolyzed casein using a Coleman Junior Spectrophotometer at 660 mμ (reported as % incident light transmitted) or by titrating the lactic acid produced in sodium caseinate media to the phenolphthalein end point (reported as ml. of 0.1 *N* NaOH per 10 ml. of culture corrected for the titration of the uninoculated control). Data given are representative of several experiments.

Results

In media containing sodium caseinate, growth of caseinate utilizing lactic streptococci was affected by the concentration of sodium acetate and this effect was influenced by the incubation time (Fig. 1). When growth was observed at 24 hours, maximum stimulation occurred at a concentration of 10 to 20 mg. of sodium acetate per ml., whereas at 40 hours, maximum stimulation occurred at 30 mg. of acetate per ml. At an acetate concentration of 40 mg. per ml. growth was less than it was at 10 to 30 mg. per ml. However, the extent of the inhibition was reduced when the incubation period was increased from 24 to 40 hours.

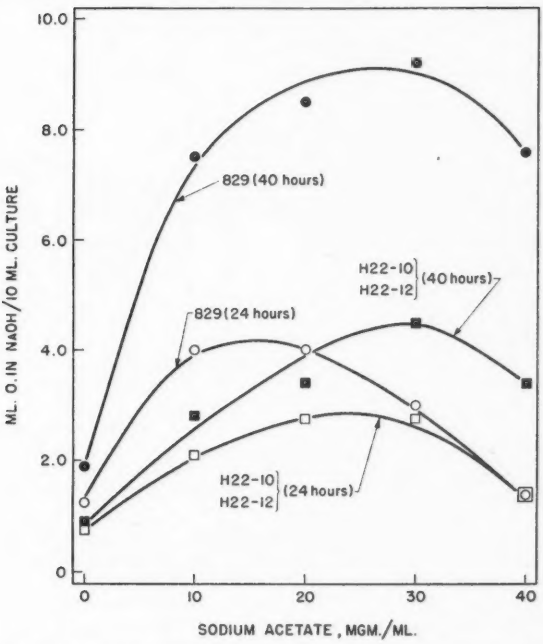

FIG. 1. Effect of sodium acetate on growth of lactic streptococci. Basal medium (text) with sodium caseinate (2.0 mg./ml.) as the nitrogen source.

In media containing 40 mg. of sodium acetate per ml., the addition of calcium reduced the inhibition by the acetate (Fig. 2). Maximum effect was obtained at about 40 μg. of added calcium per ml., and increasing the concentration to 100 μg. per ml. did not alter the results. Acetate inhibition was not overcome by the addition of 200 μg. magnesium per ml., and the addition of 800 μg. magnesium per ml. caused further inhibition of growth (Fig. 2). Since media with acetate and high concentrations of magnesium were turbid, magnesium phosphate may have precipitated.

When sodium citrate rather than sodium acetate was added to the medium, the lag phase was increased and the amount of acid produced was decreased. Growth was inhibited at a concentration of 10 mg. of sodium citrate per ml.; extending the incubation period reduced, but did not eliminate, this effect (Fig. 3). At intermediate levels of sodium citrate (2.0 to 4.0 mg. per ml.), two strains were inhibited when growth was observed at 40 hours but were stimulated slightly when growth was observed at 70 hours.

The inhibitory effect of 10 mg. of sodium citrate per ml. was relieved by the addition of calcium or magnesium to the medium (Fig. 4). The highest concentrations of magnesium or calcium tested were not inhibitory and the addition of magnesium did not result in turbidity as it did in the presence of acetate. Growth in media containing citrate and added calcium or magnesium

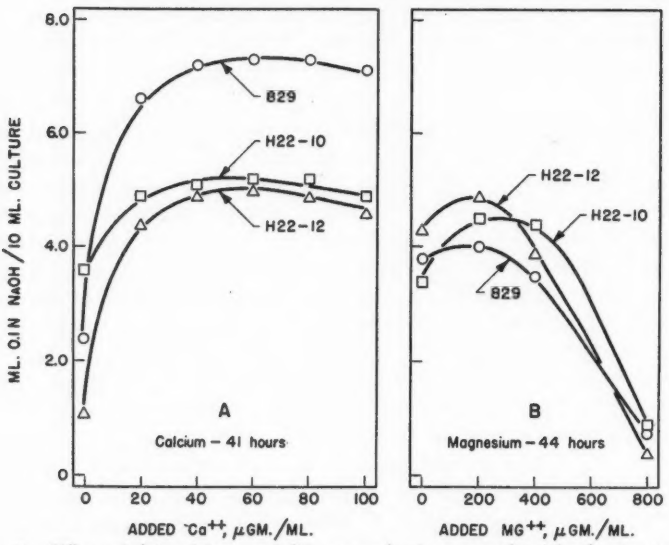

FIG. 2. Effect of (*a*) calcium and (*b*) magnesium on growth of lactic streptococci in the presence of sodium acetate. Basal medium (text) plus sodium acetate (40 mg./ml.) with sodium caseinate (2.0 mg./ml.) as the nitrogen source.

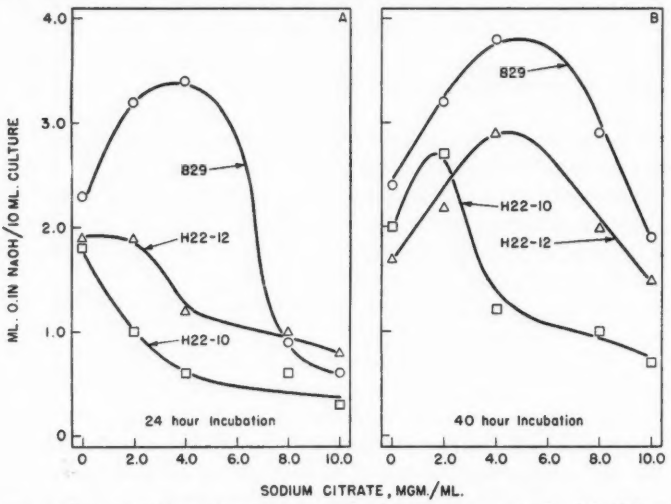

FIG. 3. Effect of sodium citrate on growth of lactic streptococci. (*a*) 40-hour and (*b*) 70-hour incubation. Basal medium (text) with sodium caseinate (2.0 mg./ml.) as the nitrogen source.

approached that obtained in media containing acetate. The inhibitory effect of citrate was not relieved by the addition in lithium (200 to 2000 μg. per ml.), potassium (200 to 4000 μg. per ml.), or barium (200 to 2000 μg. per ml.) indicating that these ions could not replace calcium or magnesium.

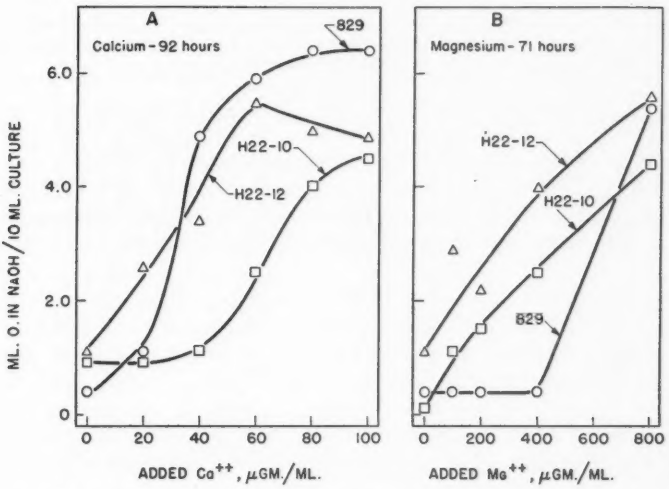

FIG. 4. Effect of (*a*) calcium and (*b*) magnesium on growth of lactic streptococci in the presence of sodium citrate. Basal medium (text) plus sodium citrate (10 mg./ml.) with sodium caseinate (2.0 mg./ml.) as the nitrogen source.

Since the nitrogen source in the above experiments was sodium caseinate, calcium and magnesium could have been activating proteases of the organisms, thus stimulating growth. However, in media containing acid hydrolyzed casein as a source of nitrogen, sodium citrate (10 mg. per ml.) inhibited growth and the addition of calcium or magnesium overcame this inhibition (Table I). Strontium did not replace calcium or magnesium although it was slightly effective during the early stages of growth of one strain. When EDTA was added to a medium containing acid hydrolyzed casein, growth of lactic

TABLE I

EFFECT OF DIVALENT METAL IONS ON GROWTH OF LACTIC STREPTOCOCCI IN MEDIA CONTAINING ACID HYDROLYZED CASEIN AND SODIUM CITRATE
(30° C.)

Addition to basal medium* (μg./ml.)	45 hr.	72 hr.	45 hr.	72 hr.	45 hr.	72 hr.
	829		H22-10		H22-12	
	% incident light transmitted					
None	41†	37†	41	34	58	37
Calcium (100)	29†	27†	35	29	38	30
None	31†	31†	47	37	51	42
Magnesium (800)	20†	20†	39	31	48	39
None	30	25	48	37	74	44
Strontium (800)	32	27	52	39	53	40

*Basal medium (text) plus acid hydrolyzed casein (*ca.* 10 mg. dry weight/ml.).
†20 mg. sodium citrate/ml.; remainder, 10 mg. sodium citrate/ml.

TABLE II

EFFECT OF CALCIUM AND MAGNESIUM ON GROWTH OF LACTIC STREPTOCOCCI IN MEDIA CONTAINING ACID HYDROLYZED CASEIN AND 1.0 m*M* DISODIUM ETHYLENE DIAMINE TETRAACETATE*

(88 hours at 30° C.)

		Added Mg^{++}, μg./ml.		
		0.0	100	200
Organism	Added Ca^{++}, μg./ml.	% incident light transmitted		
829	0.0	94	59	62
	20.0	65	53	48
	80.0	63	42	37
H22-10	0.0	100	91	91
	20.0	99	82	78
	80.0	89	72	65
H22-12	0.0	100	99	87
	20.0	75	80	77
	80.0	83	68	66

*Basal medium (text) plus 2% sodium acetate and acid hydrolyzed casein (*ca.* 10 mg. dry weight/ml.).

streptococci was inhibited (Table II). The addition of either calcium or magnesium overcame the inhibition and the effect was increased when both ions were used.

Discussion

Growth of caseinate utilizing lactic streptococci was stimulated by acetate or citrate in media with sodium caseinate as the nitrogen source, confirming observations (3, 5) of the importance of these organic acids for cultivation of lactic streptococci. However, high concentrations of sodium acetate (40 mg. per ml.) or sodium citrate (10 mg. per ml.) caused inhibition of growth. This inhibition was reduced by the addition of calcium to media containing either acetate or citrate or of magnesium to media containing citrate. In media containing soluble nitrogen, inhibition of growth by citrate or EDTA also was overcome by the addition of calcium or magnesium. These results indicate that calcium and magnesium were not concerned merely with the ability of the organisms to utilize sodium caseinate but were, in some way, essential for growth. It is possible that these metallic ions were concerned also with protein hydrolysis although protease activity in cell free extracts of *S. lactis* was not increased by metals (1, 12).

The effect of calcium and magnesium in relieving inhibition of growth by citrate and EDTA can be explained on the basis of the chelating action of these compounds. The effect of calcium in the presence of acetate is more difficult to explain. Sodium and calcium are known to be antagonistic in higher organisms and it is possible that calcium relieved an inhibition caused by the relatively high sodium content of the medium (7.0 mg. of sodium ion contributed by 40 mg. of sodium acetate per ml. plus that from sodium caseinate and sodium hydroxide used in neutralization).

The data presented suggest that calcium and magnesium stimulated growth of lactic streptococci. Many microorganisms have been shown to require magnesium and data are accumulating that indicate calcium requirements may not be uncommon. Calcium stimulated growth of *Staphylococcus pyogenes* (11) and *Clostridium perfringens* (10) in media deficient in minerals and stimulated early growth of *Lactobacillus casei* in an amino acid medium (4). *Lactobacillus delbrueckii* required calcium for utilization of serine, the requirement being increased when threonine was present (9), and in media containing limiting amounts of serine, calcium stimulated *L. casei* (2). However, *Lactobacillus arabinosus*, *Leuconostoc mesenteroides*, and *S. faecalis* did not require calcium (9).

In a study of citrate and acetate effects on growth of lactic streptococci, Kizer and Speck (5) showed that the response of *S. lactis* strains was greater with citrate than with acetate whereas strains of *S. cremoris* exhibited the reverse relationship. These authors speculated that differences in the carbohydrate metabolism might account for the effects observed. However, in the light of the above results, it seems possible that the response of these organisms to citrate and acetate might also be related to their calcium and magnesium requirements.

Acknowledgment

The author wishes to thank Dr. C. K. Johns and Mr. G. B. Landerkin for cultures and Mrs. Barbara F. Gregory for valuable technical assistance.

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THE ISOLATION OF PLEUROPNEUMONIA-LIKE ORGANISMS FROM TWO CASES OF POLYARTHRITIS[1]

C. A. BUTAS

Abstract

Two strains isolated from two cases of recurrent polyarthritis are discussed. The organisms are antigenically related and according to morphology and growth requirements they are considered to belong to the group of pleuropneumonia-like organisms.

Introduction

It has come to be realized that organisms of seemingly numerous species, some pathogenic and some not evidently so, with common properties which distinguish them from bacteria, rickettsiae, and viruses, should be included in a group called the Pleuropneumonia Group. Contagious bovine pleuropneumonia was recognized by Pasteur to be due to a specific infective agent which could not be seen under the microscope and did not grow in ordinary media. At least four different names were given to the first organism isolated: *Asterococcus mycoides* (Borrel, 1910), *Coccobacillus mycoides peripneumoniae* (Martzinovski, 1911), *Mycoplasma peripneumoniae* (Novak, 1929), *Asteromyces Pleuropneumoniae bovis* (Wroblewski, 1931). In 1898 Nocard and Roux (33) were the first to succeed in growing the agent in a cell-free medium. Its morphology was described by Bordet in 1910 (1) and also by Borrel *et al.* (2, 4), who named it *Asterococcus mycoides.* Bridré and Donatien in 1923 (3) isolated a similar organism from contagious agalactia of sheep, which, later, Wroblewski (39) named *Anulomyces agalactiae.* The morphology, cultural characters, and biochemical properties of these cultures were investigated by Holmes and Pirie (19), Ledingham (31), and Klieneberger (20).

Because the organisms seemingly passed through a Berkefeld V filter and gradocol membranes they usually were regarded as viruses, but for nearly 25 years they have been placed amongst bacteria because they were capable of multiplication in cell-free media.

In 1934 Shoetensack (see (13)) isolated another similar organism from dogs and subsequently several similar organisms were isolated from different sources (10, 18, 24, 32). Because of their resemblance to the pleuropneumonia organism they were called "pleuropneumonia-like organisms" and abbreviated to PPLO. Klieneberger (21) called L_1 an organism that she isolated from cultures of *Streptobacillus moniliformis* and which at first she considered as an associated symbiont, but later (28) she agreed with Dienes (7) that it was a variant of the bacillus. Klieneberger and Steabben (26, 25, 24), Findlay *et al.* (17), and Sabin and Johnson (35) all isolated strains from rats or mice. Sewage and soil have yielded similar though saprophytic strains.

[1]Manuscript received November 16, 1956.
Contribution from the Department of Bacteriology and Immunology, McGill University, Montreal, Que.

Dienes in 1940 (5) and others later (27) have shown that organisms of the group inhabit the human genital tract. Swift and Brown (37) claimed to have isolated pleuropneumonia-like organisms from cases of rheumatic fever but further investigations and subsequent attempts at isolation were negative. Kuzell and Mankle (30) have isolated PPLO from joint fluids in five cases of Reiter's syndrome.

Organisms isolated from two cases of acute recurrent polyarthritis are the object of this report.

Clinical Aspects

Case No. 1/H.M. age 42, female.—Her past illness revealed a rheumatic condition starting at the age of 7, and she claims that she has never been free of pain since. After her marriage, in 1943, she had the second attack of flitting polyarthritis, preceded by a marked vaginal discharge. The arthritis was diagnosed as rheumatic fever. The third attack was in 1946. The fourth attack in 1954 was preceded again by a vaginal discharge. The bacteriological examination of the vaginal discharge revealed unidentified micrococci, *Staphylococcus pyogenes*, *Paracolobactrum coliforme*, *Streptococcus fecalis*, and unidentified Gram-positive aerobic rods. The present illness began with a slight pain in the left hip, which was intermittent for a period of 1 week. It disappeared and was followed by pain and swelling of the right knee, accompanied by warmth and redness of the joint. In a few days the left knee also became swollen and painful. She had fleeting pains in the metacarpal joints of the left hand. The temperature was 101–102° F. for 2 to 3 weeks.

Case No. 2/W.C. age 35, female.—She had the usual childhood infections, except for scarlet fever and diphtheria. The patient was perfectly well until April 1953, when she had a mild sore throat, without fever or serious disability. After a week she felt unwell and noted a temperature of 100° F., but no sore throat. Five days later, she noticed a bruise the size of a 50 ct. coin above the left knee cap. Four days later the knee was stiff and the severe pain in this joint was made worse by movement. The following day there was effusion into the joint, but the X-ray showed no lesions. The diagnosis was rheumatic fever. One month later she started to have flitting pains in the right ankle, shoulder, and hip. Some joints cleared up but the left knee remained stiff, swollen, tender, and warm. Systemic symptoms have been persistent fatigue and lassitude. The temperature did not exceed 98.8° F. The second attack was in April 1954, the third in August 1954, and the present state started in November 1954.

Bacteriology

In both cases the specimens examined were aspirated knee-joint fluid, and aerobic and anaerobic cultures gave no growth of ordinary bacteria. The cultures for *Mycobacterium tuberculosis* as well as guinea-pig inoculations were negative. However, in the first case, from two consecutive specimens of articular fluid sent at a 3 day interval and cultured by two different persons

in the same way, a peculiar light growth was obtained after 5 days' aerobic incubation at 37° C. On ascitic agar slopes and plates, very small deep colonies appeared, with a brown center, visible under the microscope with a low magnification. Smears made from the deep colonies of the two specimens showed very polymorphic Gram-negative streptobacilli, staining faintly as rings, globules, and filaments. From a 10-day-old ascitic agar slope of the first specimen, agar cuts were placed into 20% ascitic broth, and, after 7 days' incubation, a slight granular sediment appeared. Smears from the sediment were difficult to interpret, because with Gram's stain they did not reveal any formed elements suggesting the presence of microorganisms, and Giemsa's stain showed only small round bodies. Subcultures made from this on solid media did not grow, but succeeded in fresh fluid medium. The original first subculture, after 14 days' incubation, showed increased granular deposit at the bottom and on the sides of the tube. Films stained with Giemsa showed polymorphic entities in the form of small and larger globules, filaments, and some coccobacillary forms. From the first subculture at this stage (14 days) we obtained again minute, deep colonies on ascitic agar plates resembling those of the primary culture. Agar cuts from the plate were used for a successive subculture and for stained impression films of the colonies. The morphology of the organism grown on solid medium consisted mostly of large masses, containing deeply staining bodies or large globules. Systematic subcultures at intervals of 4, 7, and 10 days, and even "blind passage", were continued and it was noticed that the morphology was variable with the age of the culture, and so was the rate of growth in the subculture. In our experience, the best time for subculture was between 6 and 10 days. We tried different fluid media enriched with horse serum or 20% ascitic glucose broth, before using Difco PPLO medium. Finally, after 3 months, a sufficient amount of culture in fluid medium was obtained to be used as an antigen for rabbit immunization; sufficient growth was never obtained on solid medium. The technique described by Klieneberger (23) was followed for the preparation of the antigen, as well as for the inoculation of rabbits. The serum obtained from rabbits had an agglutination titer of 1/320. In the second case (W.C.) we proceeded in the same way, but obtained growth more easily, possibly because we used the special Difco PPLO broth and PPLO agar medium. In the PPLO broth a turbidity appeared after 5 days' incubation, and the first subculture on solid medium was made after another 5 days. The microscopic and cultural aspect, as well as the impossibility of obtaining successive subcultures on solid media, was the same in both cases. Only after numerous subcultures in fluid media did films stained with Giemsa in the first case show rod-shaped forms besides the globules and round bodies of different sizes. This culture gave a heavier sediment, but even from this stage, growth on solid medium could not be obtained.

Blood serum was obtained from patient W.C. and agglutination tests were done against both strains with it, as well as with rabbit serum prepared with the culture of case H.M. The agglutination tests were done by the method

of Klieneberger, at 52° and 37° C. The rabbit serum proved to react better with both heterologus and homologus antigens than did the human serum, for which incubation at 52° C. was necessary.

	Rabbit serum prepared with H.M. culture		Serum from case W.C.	
	37° C.	52° C.	37° C.	52° C.
Antigen H.M.	1/320	1/320	0	1/40
Antigen W.C.	1/160	1/160	1/40	1/40

From the agglutination readings it appears that the organisms isolated in both cases are at least closely related. This agrees with the morphology and growth characters seen in the cultures. The exact identity of the organism is difficult to determine, but it is interesting that they were obtained from joint fluid in pure culture, from cases of active arthritis closely similar in clinical character.

Discussion

According to Sabin (36) the criteria which should decide the admission of an organism into the pleuropneumonia group are: (1) growth in cell-free media with development of polymorphic structure, including rings, globules, filaments, and filtrable elementary bodies, usually 125–250 mμ in size, which are the minimal reproductive units; (2) the development on suitable solid media of characteristic minute colonies which may be as small as 10–20μ and as a rule not larger than 600 μ. But as Dienes (8) has pointed out, this definition could include the L-phase variants of bacteria. Many different species of bacteria, grown under unfavorable cultural conditions, produce an L-phase which closely resembles organisms of the pleuropneumonia group in morphology and appearance of colonies, and it must be noted that the first L-phase organism was originally regarded by Klieneberger (22) as a PPLO. Tuslane (38) believed that the L-phase organism was indistinguishable from the pleuropneumonia group, and Dienes and Weinberger (9), while admitting the existence of an independent group of PPLO, also suggested that PPLO had evolved from bacteria by variation to an L-phase which had become stable and persistent and that this change represents a simplification in structure. However, Edward (11, 12) suggested that the L-phase organisms ought to be excluded from the pleuropneumonia group proper by colonial appearance, requirements, and possibly even by morphology. Ørskov (34), as well as Klieneberger-Nobel (28), also described morphological differences between L-phase and PPLO. In the opinion of Edward (13) the colonies of the L-phase are more opaque and their surface more heavily marked, which is recognizable at all stages of growth on examination both by naked eye and with low magnification.

The first few subcultures of an L-phase organism are difficult and often unsuccessful, but those of pleuropneumonia organisms are easier on suitable media. The PPLO are Gram-negative, but are not easily seen on films stained by Gram's method, and the cultures contain small particles, larger round bodies of different sizes, and filaments. According to Edward (14) and Freundt (16) the general properties of the organisms of the pleuropneumonia group as well as pleuropneumonia-like organisms may be summarized as follows:

1. Growth in cell-free culture media exacting special nutritional requirements for most of the species.
2. Peculiar mode of reproduction characterized by breaking up of filaments (with more or less pronounced tendency to true branching) into coccoid elementary bodies.
3. A marked tendency to pleomorphism depending on cultural conditions.
4. Characteristic appearance of the minute colonies on solid media.
5. Filtrability of the smaller reproductive units.
6. Poor affinity for the ordinary bacterial stains.
7. High resistance to penicillin and sulphathiazole.

All parasitic strains of the pleuropneumonia group require media heavily enriched with serum or ascitic fluid, but so do the L-phase variants of Gram-negative bacteria. Cholesterol seems also to be necessary for the growth of PPLO but not for the L-phase. However, some strains of the pleuropneumonia group, as well as of the L-phase variants, are unable to grow in primary culture unless another growth factor (such as liver extract, yeast extract, peptic digest of red cells, bovine albumin fraction V, lipid fraction) besides cholesterol is supplied and this factor is not always the same. Dienes (6) drew attention to short rod-shaped forms often showing bipolar staining, due to filament segmentation, and emphasized the likeness to rod-shaped bacteria. There are thus similarities in growth requirements and in morphology which support the notion that organisms of the pleuropneumonia group and L-phase organisms may be closely related, and which make it difficult to differentiate between them. It is claimed that the most characteristic property of all these organisms is that they have no rigid cell wall, and this probably accounts for their similarities and the structure of the colonies. In a recent review Edward and Freundt (15) point out again some dissimilarities between PPLO and L-phase organisms: the filtrability of PPLO and the formation of "elementary bodies" in filamentous structures, but this latter characteristic, in the opinion of Dienes (8), can be used only with great caution for taxonomic purposes, while filamentous growth mostly occurs in the L-phase.

Streptobacillus moniliformis (Levaditi *et al.* 1925) is found in the respiratory tract of rats (26) but when introduced into the skin or alimentary tract in human beings it invades the blood stream with subsequent localization in various parts of the body, most frequently in the joints. Pleuropneumonia-like organisms occur in cattle, rats, mice, etc., as hosts, and have been found in

the normal and diseased human genital tract. Recently Kuzell and Mankle (30) reported five cases of Reiter's syndrome with the isolation of PPLO from joint fluids. *S. moniliformis* grows under aerobic conditions in 2–3 days; the colonies are raised and granular and measure 3–5 mm. in diameter. After 2–4 days' incubation, microscopic colonies of the L-variant can be found buried in the agar beneath each large colony. These L-phase colonies can be maintained by serial subcultures in fluid medium, and it is possible to recover growth of streptobacilli from the L-variants. However, Klieneberger and Hellman were able to obtain several L-strains which would not revert to the streptobacillary form. The PPLO, in contrast, gives minute colonies which may require 2–4 days' growth before they can be detected with the aid of magnification. Isolated colonies appear discretely, slightly elevated with either a nipple-like or a darker center. They are 10 to 600 μ in size, depending on the species and conditions of growth. Growth in fluid medium can be obtained in a few days by dropping cutout pieces of agar containing a colony into tubes. It appears as a granular growth close to the piece of agar and rarely as diffuse turbidity. These differences and other arguments favor the supposition that in our cases we were dealing with PPLO cultures. No culture in either case resembled those described for *S. moniliformis*, either on agar or in fluid media. The primary cultures obtained from the joint fluid in our cases were minute deep colonies diffusing into the agar, difficult to subculture, and giving a granular growth in fluid medium. Morphologically they were polymorphic, rod-shaped forms developed late, which is characteristic of PPLO cultures, depending on the age and evolution phase of the organism. In older cultures rod-shaped forms appeared, again characteristic of PPLO, this being due to filamentous fragmentation. On the other hand, we know that the L-phase of *S. moniliformis*, whether considered as a PPLO or as one of symbionts, L^1 or L^2 (21), cannot be separated from the bacterial strain by the usual methods, but only after the heating of old cultures at 52–53° C. for 5 to 15 minutes. We did not obtain any growth after the heating or after filtration of our cultures.

The growth requirements which seem to be important for the differentiation of PPLO from the L-phase of bacteria also favor our hypothesis, because our strains did not grow without heavy serum enrichment, which supplied the cholesterol, an essential factor for PPLO. Further tests are required for the exact identification of the cultures from these two cases, especially agglutination typing with pleuropneumonia sera, which are not at our disposal, or serum neutralization, because there appear to be certain similarities between viruses and PPLO. The injection of hyperimmune serum into an animal before it is inoculated with a pathogenic strain of PPLO prevents infection. Growth is also prevented by incorporating an antiserum in the medium.

We believe that we can exclude the possibility of *S. moniliformis* infection and consider that these cases are PPLO infections. It might be considered necessary to exclude the possibility of a permanent L-phase. Edward does not include the L-phase in the pleuropneumonia group but some other authors

believe that the L-phase is sometimes indistinguishable from an organism of the pleuropneumonia group, and, much more, they suggest that PPLO have evolved from bacteria by variation to a stable and persistent L-phase, representing a simplification of structure.

Acknowledgment

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SILICON METABOLISM IN DIATOMS

IV. GROWTH AND FRUSTULE FORMATION IN NAVICULA PELLICULOSA[1]

JOYCE C. LEWIN[2]

Abstract

The cells of *Navicula pelliculosa* (Bréb.) Hilse, when grown in media which contained 3.5, 8.3, and 34.0 mg. Si per liter, reproduced with generation (doubling) times of 20.4, 13.7, and 13.1 hours respectively. The degree of silicification of the cells in each culture initially fell with increasing population. After exponential growth had ceased, this value varied between 0.8×10^{-9} and 2.2×10^{-9} mg. Si per cell, depending on the amount of silicon originally present in the medium and on the age of the culture. When placed in a fresh silicate solution, the cells took up additional silicate with increases in silicon content of 50%, 90%, and 15%, respectively. Silica (SiO_2) may represent from 4% to 22% of the dry weight of the cells. The average thickness of the frustules has been calculated to range between 200 and 600 Å.

Introduction

Einsele and Grim (1) calculated the silicon content of the cells in 15 species of planktonic fresh-water diatoms collected in nature. Even within a given species they found that the silicon content varied widely, depending upon the depth at which the cells had been collected and the stage of the growth cycle. Lund (6) found variation in the silica content of *Asterionella formosa* collected in nature, and noted that in cultures, as the cell number increased, the amount of silica per cell was reduced. Jørgensen (3) likewise reported that the amount of silica in the walls of *Nitzschia palea*, *Bacillaria paradoxa*, and *Thalassiosira nana* was inversely proportional to the number of cells in the culture.

Since many species of diatoms are known to decrease in size as a result of their special mode of vegetative division, one could attribute a reduction of silicon content simply to a decrease in the area of the frustules. For this reason, experiments were undertaken with *Navicula pelliculosa* (Bréb.) Hilse, a diatom which is known not to decrease in size. Cells grown with a limiting supply of silicon are capable of absorbing more silicon when transferred to a silicon-rich medium, even under conditions where cell division cannot occur (4). This suggests that the amount of silicon per cell may be variable in this species. To determine the extent of this variation, cells were grown in media with various silicon contents, and the amount of silicon in the cells determined periodically during the growth period. The results are reported in this paper.

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Contribution from the Atlantic Regional Laboratory, National Research Council, Halifax, Nova Scotia, Canada.
Issued as N.R.C. No. 4230.
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Materials and Methods

The mineral medium employed contained the following concentrations of salts (w/v): K_2HPO_4, 0.02%; $MgSO_4.7H_2O$, 0.02%; $Ca(NO_3)_2.4H_2O$, 0.1% in distilled water. Traces of B, Mn, Zn, Cu, Co, Mo, and Fe were added. Cells were grown in three 2-liter Pyrex Erlenmeyer flasks containing 1.5 liters of medium. Potassium silicate was added to flask A to give a total silicon concentration of 3.5 mg. Si per liter; to flask B, 8.3 mg. per liter; and to flask C, 34.0 mg. per liter. Each flask was inoculated with 1 ml. of a uniform suspension containing 1.25×10^7 cells. The cultures were constantly aerated by filtered air and illuminated with white fluorescent lights (300 ft-c.) at 23° C.

At intervals a 9 ml. sample was removed from each flask under sterile conditions. Cell densities were determined by use of a hemacytometer. The cells were then removed by centrifugation and the soluble silicon remaining in the supernatant was determined colorimetrically. The cells were treated with 2 ml. 1% HCl to dissolve any precipitated calcium silicate and phosphate and washed with 3 ml. distilled water. Acid and water washings were combined for determination of the "insoluble" silicon in the growth medium. Silicon was determined colorimetrically in the "soluble" and "insoluble" fractions by the molybdate reaction (4).

To determine the silicon in the frustules of each sample, the washed cell residues were transferred to platinum crucibles, evaporated to dryness, and ashed by heating to red heat. When cool, a solution of Na_2CO_3 was added in the following amounts: 0.3 mg. to the samples from flask A, 0.8 mg. to those from flask B, and 3.4 mg. to those from flask C. The samples were then fused by heating to redness. The sodium silicate so formed was extracted with water to a total volume of 9 ml., and the soluble silicon determined colorimetrically.

At the end of the growth period, silicon in the cells was determined gravimetrically. Aliquots containing a known number of washed cells were dried at 100° and weighed, ashed, heated with concentrated H_2SO_4, and dried and weighed again. They were then heated with HF + H_2SO_4 to remove silicon as volatile SiF_4. After treatment with HF the ash was weighed once more, and from the loss in weight the SiO_2 originally present in the cells was calculated.

On the 15th day, the cells were tested for their ability to take up silicon under conditions in which no cell division occurred (4). Cells removed from the growth medium were resuspended in a fresh silicate solution (12 mg. Si per liter) at a density of approximately 10^7 cells per milliliter, and incubated in darkness until no more silicon was removed from the solution (see (4)).

Results

1. Growth and Silicon Utilization

The number of cells per milliliter in each flask has been plotted against time (Fig. 1). The generation (doubling) times have been found to be 20.4 hours in flask A (3.5 mg. Si per liter), 13.7 hours in flask B (8.3 mg. Si per liter), and 13.1 hours in flask C (34.0 mg. Si per liter).

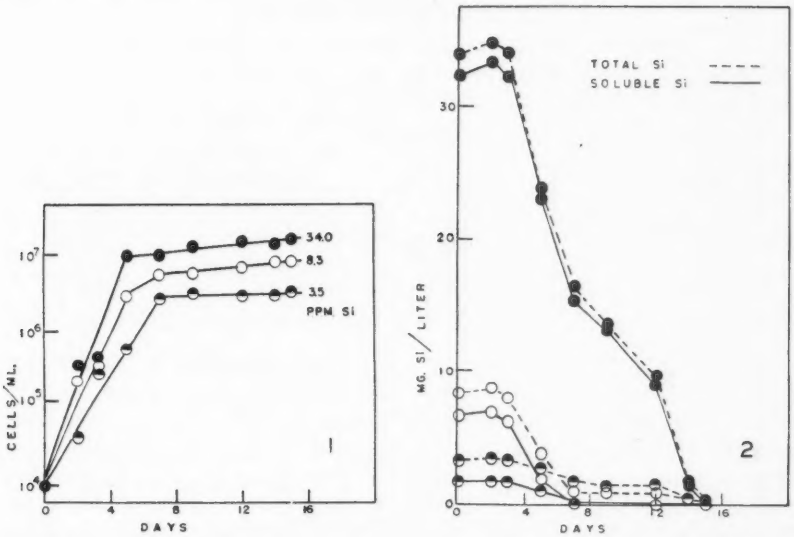


FIG. 1. Growth of *Navicula pelliculosa* in media originally containing 3.5, 8.3, and 34.0 p.p.m. Si.

FIG. 2. Amount of Si (total and soluble) present in the medium during growth period of diatom cells. (Symbols correspond to those in Fig. 1.)

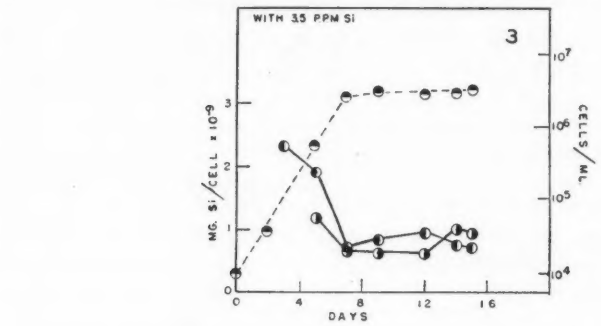
The amounts of silicate (total and soluble) remaining in the media have been plotted in Fig. 2. The insoluble silicate precipitated on autoclaving was independent of the total silicon present, being 1.65 mg. Si per liter in flask A, 1.76 mg. Si per liter in flask B, and 1.50 mg. Si per liter in flask C. At the end of the experimental period, 0.3 to 0.4 mg. insoluble Si per liter remained undissolved and unused by the diatoms.

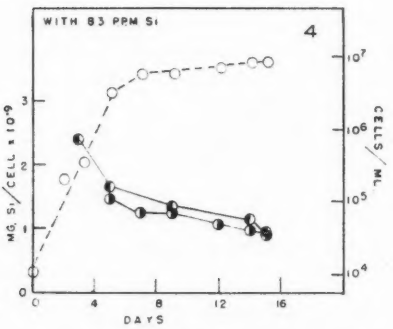
In flasks A and B all soluble silicate had disappeared by the 7th day, corresponding to the time when exponential growth ceased (see Fig. 1). In flask C, since much silicate remained, some other factor had become limiting for growth.

2. Amount of Silicon Per Cell

The weights of silicon per cell, as determined by direct measurement and by difference, are shown graphically in Figs. 3, 4, and 5. For each flask the growth curve has also been shown.

The degree of silicification of the cells in each flask initially fell with rising population. After exponential growth had ceased, the silicification depended upon the amount of available silicon still present in solution. In flask A, the silicon content of the cells became constant at about 0.8×10^{-9} mg. Si per cell. In flask B, the silicon content decreased from 1.5×10^{-9} to 1.0×10^{-9} mg. Si per cell, since slow cell division continued between the 7th and 15th days. In flask C, there was still abundant silicate present





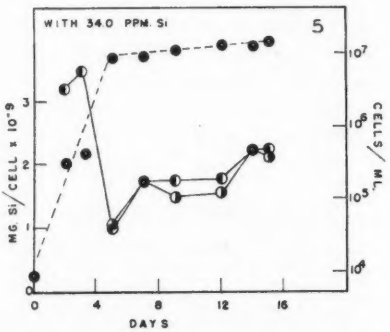


FIG. 3. Amount of Si per diatom cell as determined by direct measurement (◐) (Na_2CO_3 fusion method) and by difference (◑) (loss from the medium). ◓ = Number of cells per milliliter. Culture grown with 3.5 p.p.m. Si.

FIG. 4. Amount of Si per diatom cell as determined by direct measurement (◐) (Na_2CO_3 fusion method) and by difference (◑) (loss from the medium). ○ = Number of cells per milliliter. Culture grown with 8.3 p.p.m. Si.

FIG. 5. Amount of Si per diatom cell as determined by direct measurement (◐) (Na_2CO_3 fusion method) and by difference (◑) (loss from the medium). ● = Number of cells per milliliter. Culture grown with 34.0 p.p.m. Si.

after exponential growth had ceased, and during the remaining phase of slow cell division the silicon content of the cells increased from 1.0×10^{-9} to 2.2×10^{-9} mg. Si per cell. On the 15th day, the amounts of SiO_2 per cell, determined by three different methods, were in close agreement (Table I).

3. *Uptake of Silicon by Non-dividing Cells*

On the 15th day, samples of the cells were transferred from the nutrient media to a solution containing about 12 mg. Si per liter. After 24 hours, when the cells had ceased to take up any further silicon from the solution, the additional silicon uptake per cell was calculated. The values obtained were: flask A, 0.43×10^{-9} mg. per cell; flask B, 0.87×10^{-9} mg. per cell; flask C, 0.36×10^{-9} mg. per cell. These values correspond respectively to increments in silicon content of approximately 50%, 90%, and 15%.

TABLE I

CONTENT OF SILICA IN *N. pelliculosa* AFTER 15 DAYS OF GROWTH

Original conc. of Si (p.p.m.) in medium	3.5	8.3	34.0
Dry weight of single cell (mg. $\times 10^{-9}$)	47	26	22
Percentage of SiO_2 determined by:			
(*a*) removal of Si from medium	4.3	7.5	21.4
(*b*) fusion of cells with Na_2CO_3	3.2	7.4	21.9
(*c*) treatment of cells with HF	4.3	11.2	27.9
Length of cell (μ):			
Average	8.6		8.1
Range	7.2–9.3		7.2–9.3

4. *Thickness of the Valve*

Since no consistent differences in the general shape of the cells have been observed, and since the cell length appears to be unaffected by the silica content of the medium (see Table I), an estimate of the average valve thickness can be made in the following way. The silica frustule of *Navicula pelliculosa* consists of a pair of flat elliptical valves with dimensions of $8\mu \times 3\mu$, and a combined area of 40 μ^2. Since the siliceous frustules in this species contain 9.6% bound water (kindly determined by Dr. V. T. Bowen, Woods Hole Oceanographic Institution), their specific gravity would be rather lower than that of anhydrous silica. It may be taken as 2.0 for the present purpose.

Cells grown in media of low silicon concentration contained 0.8×10^{-9} mg. Si per cell, equivalent to 1.7×10^{-9} mg. SiO_2 per cell. The frustular volume of a single cell would therefore be 0.85×10^{-9} mm.^{3} = 0.85 μ^3, and the average thickness of the valve 0.85 μ^3/40 μ^2 = 0.02 μ = 200 Å.

Similarly, cells grown in high silicon concentration, containing 2.2×10^{-9} mg. Si per cell, would have an average valve thickness of 0.06 μ = 600 Å.

Electron micrographs of acid-cleaned valves (Fig. 6) reveal a pattern of pores in this, as in other diatoms. Moreover, the valve thickness is far from uniform, being greatest in the region of the raphe, and least at the margins, where the silica appears to be pliable and partly transparent to the electron beam. From photographs of shadowed preparations (Pd, 1:7) (Fig. 7), estimates of marginal thicknesses between 30 and 100 Å were obtained.

The valve thickness was also measured in electron micrographs of sections of whole cells and found to be of the order of 300–500 Å.

Discussion

In *N. pelliculosa* the silica of the frustule constituted from 4 to 22% of the dry weight of the cell at the end of the experimental growth period of 15 days. The percentage is influenced by the original concentration of silicon in the culture medium. As shown in Table I, the dry weight of a single cell grown in a medium of low silicon content (3.5 p.p.m.) was twice that of one grown in a medium of high silicon content. This difference is

explicable by the fact that cells grown in such a medium secrete a capsule in response to continued photosynthesis without cell division (5). Some cells in the medium with 8.3 p.p.m. Si showed evidence of capsule formation. No capsules were visible around cells in the medium with 34 p.p.m. Si.

The silicon content in *N. pelliculosa*, after exponential growth has ceased, has been shown in these experiments to vary between 0.8×10^{-9} and 2.2×10^{-9} mg. Si per cell, depending on the amount of silicon originally present in the culture medium and on the age of the culture. The thickness of the frustules was in the region of 200 to 600 Å. Einsele and Grim (1) found that the frustules of various species of fresh-water pennate diatoms varied between 800 to 1500 Å in thickness. Since *N. pelliculosa* is weakly silicified, the lower values obtained for this organism are to be expected.

The cells of many species of diatoms decrease in size as a result of repeated cell division. Furthermore, Hendey (2), among others, has observed that a change in form may be associated with a decrease in size, so that small cells of a given species do not have the same geometrical proportions as the larger ones from which they arise. If the diatoms under investigation do show size variation, then for purposes of comparison it may be preferable to express the silica content as weight per unit of cell surface or cell volume. Einsele and Grim (1) stated that, with few exceptions, the fluctuations in linear dimensions of the diatom species which they observed were insignificant. Nevertheless, they took the size of the cells into consideration, and expressed their values as weight of SiO_2 per μ^2 of surface. Lund (6) mentioned that the variation in silica content may be related to variation in the size of the cells.

The results obtained with *Fragilaria crotonensis* and *Asterionella formosa* (1), with *A. formosa* (6), with *Nitzschia palea*, *Bacillaria paradoxa*, and *Thalassiosira nana* (3), and in the present study with *Navicula pelliculosa*, all demonstrate a decrease in the degree of silicification of diatoms with increasing population. Lund, like Einsele and Grim, considered that the variation in silica content depended on the growth rate. However, Jørgensen found that in *N. palea* the same variations in silica content occurred even when the organisms were growing in a flowing culture solution, in which he believed the growth rate to be constant. He therefore postulated that the decrease in silica content with increasing population might result from the secretion of a substance inhibitory to silicate assimilation. This seems improbable for several reasons. If such an inhibitor were involved, one would expect the greatest effect to be observed when the cell density was highest, whereas Jørgensen found the reverse to be true. Moreover, it appears unlikely that such a compound would accumulate in a flowing culture. Other explanations are more plausible. For instance, if the average size of the cells became smaller with repeated division, then the silicon content per cell would likewise be expected to decrease. However, Jørgensen (personal communication) found that the average cell length decreased during 8–10 divisions by only 1 μ, which he believed insufficient to account for the observed differences. The wide range of apparent Si content per cell, in Jørgensen's

PLATE I

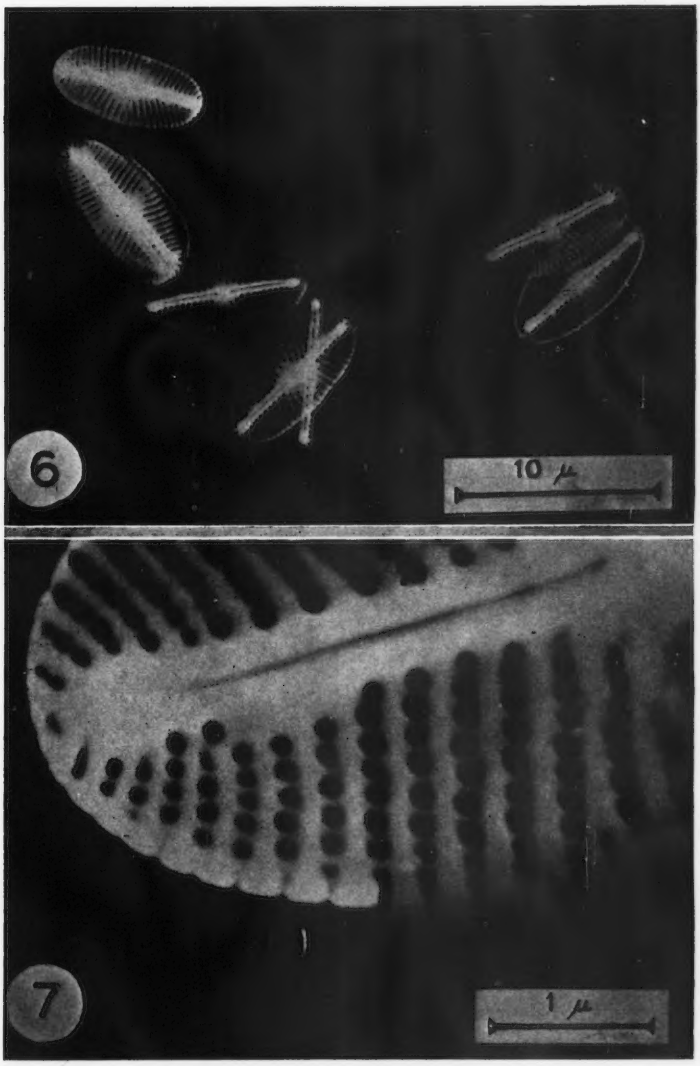


FIG. 6. Frustules of *Navicula pelliculosa.*
FIG. 7. Portion of frustule. Shadowed with Pd (1:7).

results, could be explained by an error of about 80 μg. Si per flask. This could be accounted for if there was any precipitated silicate mixed with the cells or adherent to the surface of the vessel, which would remain with the cells in the sulphuric acid which Jørgensen employed to loosen them for transfer to platinum crucibles. Until more convincing data are available, the existence of an inhibitory substance as postulated by Jørgensen must be considered doubtful.

Acknowledgments

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PHASE VARIATION OF RICKETTSIA (COXIELLA) BURNETI

STUDY OF THE ANTIBODY RESPONSE IN GUINEA PIGS AND RABBITS[1]

PAUL FISET[2]

Abstract

Further studies of the phase variation of *Rickettsia burneti* described by Stoker (12, 13) and Stoker and Fiset (14) are reported. Animals (guinea pigs and rabbits) infected or vaccinated (with killed purified suspensions) with either Phase 1 or Phase 2 of *R. burneti* (Nine Mile and Christie strains) develop antibodies that are detectable very early (within 21 days after inoculation) when their sera are tested with antigens in Phase 2. Except for the group of guinea pigs vaccinated with killed purified suspensions of Nine Mile Phase 2, all the animals also produce antibodies against the Phase 1 strains but these appear much later than the anti-Phase 2 antibodies. It is believed that *R. burneti* when reverting from the non-reactive Phase 1 to the reactive Phase 2 through egg adaptation loses some of its surface antigens.

A variation in the complement fixing activity of *Rickettsia burneti* in the course of egg adaptation was reported by Stoker (12, 13) and the phenomenon was further investigated by Stoker and Fiset (14). It will be recalled that strains isolated from their natural hosts (cows, sheep, ticks) or from guinea pigs, in which the strains had been maintained through serial passages, fail to react in the complement fixation test with human convalescent serum or with homologous guinea-pig antiserum collected up to 40 days after infection. After several passages in the yolk sac of developing chick embryos, however, rickettsial suspensions are obtained which react readily with the same antisera. The non-reactive phase occurring in the early stages of egg adaptation was called Phase 1, whereas the reactive phase occurring after several egg passages was called Phase 2. A reversal from the reactive Phase 2 to the non-reactive Phase 1 can be induced by one or more passages in adult animals (guinea pigs, mice, hamsters) of a thoroughly egg-adapted strain. The variation is completely reversible and can be induced experimentally according to the following pattern:

$$\text{Phase 1 (non-reactive)} \underset{\text{in adult animals}}{\overset{\text{in chick embryos}}{\rightleftharpoons}} \text{Phase 2 (reactive).}$$

Though suspensions of *R. burneti* in Phase 1 fail to react in the complement fixation test with homologous guinea-pig antiserum collected up to 40 days after infection, it was observed, however, that complement fixation occurred with antisera collected later.

The present study was undertaken in the hope that the antibody response to infection and vaccination (with killed purified suspension of *R. burneti*) would provide some information about the antigenic differences of the two phases.

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Materials and Methods

Strains of R. burneti

Two strains were selected for this investigation: the American Nine Mile and the British Christie.

Nine Mile Strain

This strain was isolated by Davis and Cox (4) from a pool of *Dermacentor andersoni* collected at Nine Mile Creek in Montana. It was first called *Rickettsia diaporica* (2) but its similarity to *R. burneti*, the causal agent of Australian Q fever described by Derrick (5, 6), was soon established by Dyer (7, 8) and Burnet and Freeman (1). This strain was selected because it was widely used for preparing diagnostic antigen and also because it was known that two lines of this strain existed, one guinea-pig-adapted and the other egg-adapted.

At the Rocky Mountain Laboratory in Hamilton, Montana, this strain had been maintained by serial passages in guinea pigs and when it was received from Dr. Lackman in 1953 as a lyophilized spleen suspension it had had 307 guinea-pig passages. It was inoculated once more into guinea pigs and spleen material obtained from this passage was used throughout. For preparing antigen two passages were carried out in the yolk sac of developing chick embryos. This strain will be referred to as Nine Mile-Rocky or Nine Mile Phase 1.

Shortly after the isolation of the Nine Mile strain by passage into guinea pigs, Cox (3) succeeded in adapting the organism to developing chick embryos. When this strain was received from Dr. Cox of the Lederle Laboratories in 1949 it had had between 50 and 60 egg passages and was used extensively for preparing diagnostic antigen. This strain will now be referred to as Nine Mile-Led or Nine Mile Phase 2.

Christie Strain

This strain was isolated in London by MacCallum *et al.* (9) and its characteristics have been described by Stoker (12, 13). It was isolated by inoculation into guinea pigs of blood collected from a patient in the acute phase of Q fever. The strain was passaged twice in guinea pigs and then in chick embryos. It will be recalled that Stoker (13) reported that this strain, in the course of egg adaptation, reverted from the non-reactive (Phase 1) to the reactive (Phase 2) phase at the fifth yolk sac passage. In the present investigation the third yolk sac passage was used as a source of Phase 1 material and the ninth as a source of Phase 2.

Antigens

Antigens in the form of purified rickettsial suspensions were prepared from infected yolk sacs by repeated ether extractions and differential centrifugation (16, 13). The antigens were standardized by photoelectric densitometry to match a Nine Mile-Led strain antigen chosen as a standard (S). Counts carried out on this standard antigen showed that it contained about 2×10^{13} particles per milliliter (14).

Serological Tests

Complement fixation tests were carried out by the standard technique used in this laboratory (13, 15). Two minimal hemolytic doses of complement were used. Preparations of Nine Mile-Led (Phase 2) antigen were titrated in chessboard fashion with homologous early (21 days after infection) guinea-pig antiserum, i.e., increasing doubling dilutions of antigen with increasing doubling dilutions of antiserum. The highest dilution of antigen giving the highest antibody titer was taken as one antigen unit. Throughout this investigation all the sera were tested with three units of Nine Mile Phase 2 antigen and this was found to correspond to a dilution of 1:50 in terms of our standard (S). The Christie antigens and the Nine Mile Phase 1 antigen were also used at a concentration of 1:50 S.

Experimental

Studies with the Nine Mile Strain

(1) Serological Response to Infection

Guinea pigs were used for this experiment because they are known to be very susceptible to infection with *R. burneti*. The animals were bled before infection and at regular intervals over a period of 110 days after infection and their sera were tested with antigens of both phases of the Nine Mile and the Christie strains.

(a) Infection with Nine Mile-Rocky, Phase 1

Six animals were inoculated intraperitoneally with 1 ml. of a 10^{-2} suspension of spleen from the 308th guinea pig passage of the Nine Mile-Rocky strain. The results are recorded in Fig. 1*a*. It will be noted that the Nine Mile and the Christie strains cross-react very strongly. Antibodies against both antigens in Phase 2 become detectable between the 14th and 20th day after infection. Antibodies against both antigens in Phase 1 are not detectable before the 40th day and reach a plateau by the 60th. At about 104 days after infection both anti-Phase 1 and anti-Phase 2 antibodies are at approximately the same level.

(b) Infection with Nine Mile-Led, Phase 2

Six guinea pigs were inoculated intraperitoneally with 2 ml. of the 10^{-3} yolk sac suspension of the Nine Mile-Led strain. The results are recorded in Fig. 1*b*. Here again the anti-Phase 2 antibodies are detectable between the 14th and the 20th day after infection when they reach their maximum titer. From the 30th day they decline progressively. Anti-Phase 1 antibodies are not detected before the 40th day, reach their maximum by the 50th, and begin to decline after the 70th. These antibodies never reach very high titers. Here again the Nine Mile and the Christie strains are found to cross-react very strongly.

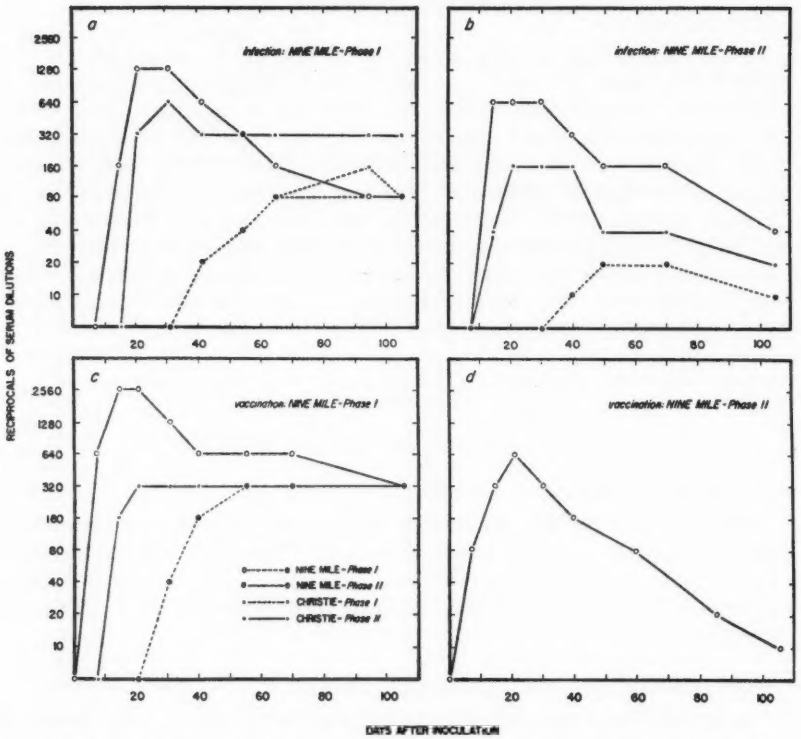

FIG. 1. Antibody response in guinea pigs, (*a*) infected with Nine Mile-Rocky, Phase 1, (*b*) infected with Nine Mile-Led, Phase 2, (*c*) vaccinated with a killed suspension of Nine Mile, Phase 1, and (*d*) vaccinated with a killed suspension of Nine Mile, Phase 2.

(2) *Serological Response to Vaccination with Purified Killed Suspensions of the Nine Mile Strain*

As shown above, guinea pigs whether infected with the Phase 1 or Phase 2 variant of the Nine Mile strain of *R. burneti* develop antibodies against both phases of the Nine Mile and the Christie strains. The Phase 2 antibodies are always detected much earlier than the Phase 1. It must be recalled that Stoker and Fiset (14) have shown that *R. burneti* reverts readily from Phase 2 to Phase 1 by passage in guinea pigs and this may explain why both groups of infected animals show a similar antibody response.

It was thought that if, instead of being inoculated with live rickettsiae that undergo the variation described by Stoker and Fiset (14), the animals were vaccinated with purified killed suspensions of organisms, a somewhat different pattern would be observed.

The experiments were carried out in guinea pigs and rabbits. The antigens used for vaccination, which had been stored in formolized (0.25%) saline,

were washed once before use and resuspended in 0.9% saline containing 1000 units of penicillin per milliliter. The antigens were diluted to match the concentration of our standard (1 S).

Vaccination of Guinea Pigs

Two groups of six guinea pigs were inoculated with Nine Mile-Rocky (Phase 1) and Nine Mile-Led (Phase 2) antigens respectively. They were given a single injection of 1 ml. intraperitoneally.

In the course of this experiment it was found that Nine Mile-Rocky was very toxic for guinea pigs and killed a large number of them within 24 hours after inoculation. The toxic effect, however, could be neutralized by normal rabbit serum (Stoker and Fiset, unpublished data). The antigen was therefore diluted 1:2 in normal rabbit serum.

(*a*) *Response to Vaccination with Nine Mile-Rocky, Phase 1*

The results are recorded in Fig. 1*c*. As expected, the antibodies appear sooner than in the infected animals. Anti-Phase 2 antibodies are detectable by the 7th day and are at their maximum by the 15th. Anti-Phase 1 antibodies become detectable between the 20th and 30th day and have reached their highest titers by the 40th. Both strains cross-react very strongly but the homologous (anti-Nine Mile) antibodies are the first ones to appear.

On the whole, the general pattern is very similar to that observed when guinea pigs are infected with Nine Mile-Rocky with the exceptions that all antibodies appear somewhat sooner and that anti-Phase 1 antibodies reach higher titers.

(*b*) *Response to Vaccination with Nine Mile-Led, Phase 2*

The results are recorded in Fig. 1*d*. Several striking features were observed in this experiment. None of the animals developed anti-Phase 1 antibodies against either the Nine Mile or Christie strain. Only two produced anti-Christie Phase 2 antibodies while the others did not. These antibodies were of low titer, appeared by the 20th day, and were no longer detectable after the 40th.

All the animals produced anti-Nine Mile Phase 2 antibodies that reached a peak by the 15th day and declined progressively after that time. The decline was fairly rapid and in one of the animals antibodies were no longer detectable after the 85th day. The Nine Mile Phase 2 antibody titers were, on the whole, somewhat lower than those found when the vaccination was carried out with Nine Mile Phase 1.

Vaccination of Rabbits

Two groups of two rabbits were inoculated respectively with Phase 1 and Phase 2 antigens of the Nine Mile strain. The animals were given three injections of standard concentration. The first injection, 1 ml., given intravenously, was followed by two of 0.5 ml. given subcutaneously at 2 day intervals. The animals were bled regularly up to 125 days after the first injection when they were reinoculated with 1 ml. of the same antigen given

subcutaneously. They were then bled weekly for a period of 35 days and their sera were tested with antigens of both phases of the Nine Mile and Christie strains.

(*a*) *Response to Vaccination with Nine Mile-Rocky, Phase 1*

The results obtained were very similar to those observed in guinea pigs vaccinated with the same strain with the exception that all titers were higher (Fig. 2*a*). Nine Mile and Christie anti-Phase 2 antibodies reach a peak by the 15th day. They drop slightly to reach a plateau by the 45th day and remain at this level until the 125th day. Anti-Phase 1 antibodies appear

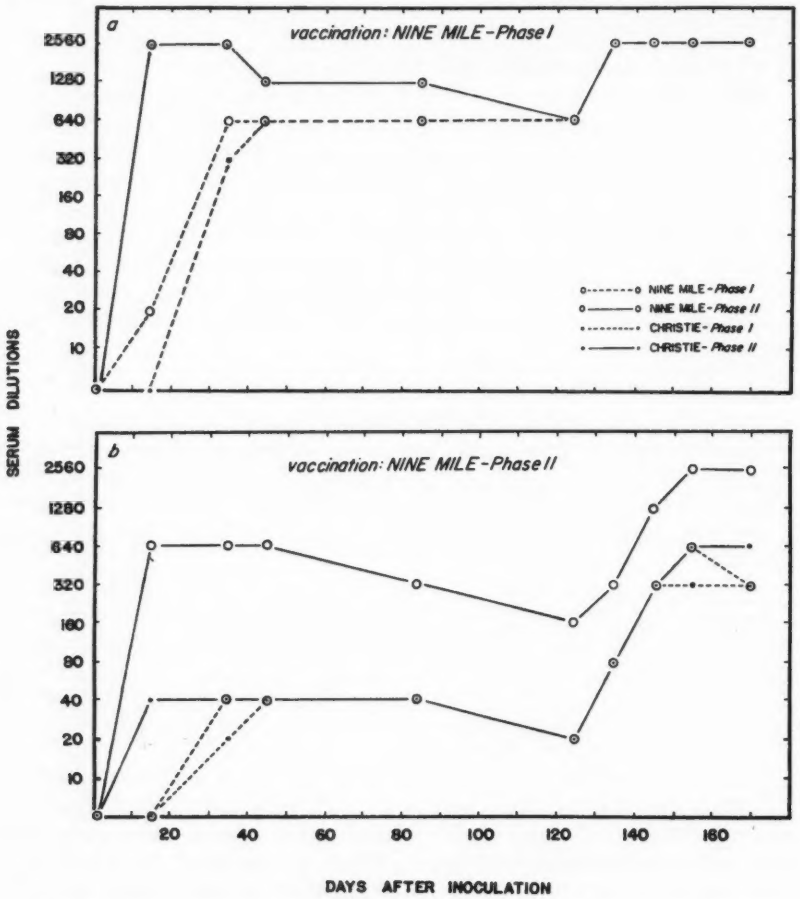

FIG. 2. Antibody response in rabbits vaccinated with killed suspensions of (*a*) Nine Mile-Rocky, Phase 1, and (*b*) Nine Mile-Led, Phase 2.

later and are at their highest by the 35th day. They remain more or less at this level until the 125th day. From the 35th to the 125th day Phase 1 and Phase 2 antibodies are roughly at the same level.

On the 125th day the animals were reinoculated with 1 ml. of Nine Mile-Rocky antigen. There was a sudden rise in all the antibody titers that reached a plateau 14 days after the booster injection and remained at that level until the end of the experiment. The titers reached by all the antibodies after challenge were as high as the original peak of the Nine Mile Phase 2 antibodies.

(*b*) *Response to Vaccination with Nine Mile-Led, Phase 2*

The vaccinated rabbits were bled on the same days as those of the previous experiment and their sera were tested with the same antigens.

The results recorded in Fig. 2*b* show that Nine Mile Phase 2 antibodies reach high titers and are at their maximum by the 15th day. After this time they drop slowly until the 125th day. The Christie Phase 2 antibodies also attain their maximum by the 15th day but never reach high titers. In one rabbit the difference in titers at the highest point between the Christie and Nine Mile Phase 2 antibodies was fourfold (Fig. 2*b*), while in the other one it was sixfold.

Both the Nine Mile and the Christie Phase 1 antibodies are at their maximum by the 35th day and remain at this level until the 125th day. They never reach titers higher than those of the Christie Phase 2 antibodies.

When the animals were reinoculated on the 125th day they showed a sudden rise in all their antibody titers that reached a maximum 20 days after challenge and remained at this level until the end of the experiment. This level was almost as high as the original peak attained by the Nine Mile Phase 2 antibodies.

Studies with the Christie Strain

The following experiments with the Christie strain were carried out in order to compare the serological behavior of this strain with that of Nine Mile. It was felt that it would be sufficient to limit the investigation to the serological response to vaccination in guinea pigs and rabbits. The procedures followed for inoculating, bleeding, and testing were identical with those used in the Nine Mile experiments.

(*1*) *Serological Response to Vaccination in Guinea Pigs*

Two groups of five guinea pigs were inoculated with killed purified suspensions of Christie Phase 1 and Phase 2 respectively. The results are recorded in Fig. 3*a* and *b*. No significant difference seems to exist between the two groups. There is a sharp and early rise in Phase 2 antibodies with Nine Mile antibodies reaching higher titers. The titers decline progressively and reach a plateau by the 70th day.

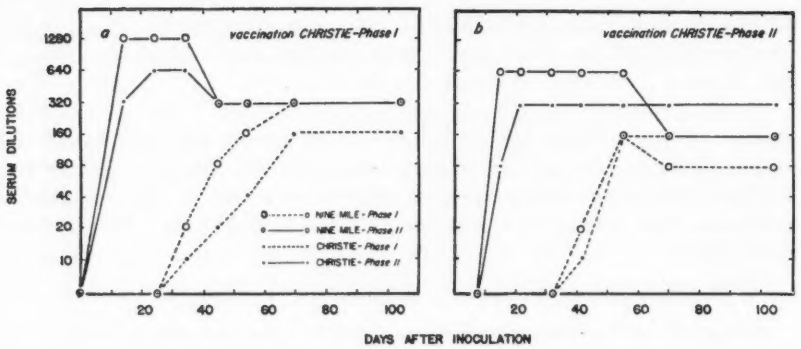

FIG. 3. Antibody response in guinea pigs vaccinated with killed suspensions of (*a*) Christie, Phase 1, and (*b*) Christie, Phase 2.

Phase 1 antibodies become detectable by the 30th day and rise progressively until, by the 70th day, they have reached a plateau at the same level as the Phase 2 antibodies.

On the whole, the general pattern obtained with both phases of the Christie strain is very similar to that observed when guinea pigs are vaccinated with Nine Mile Phase 1.

(*2*) *Serological Response to Vaccination in Rabbit*

Two rabbits were inoculated with killed purified suspensions of Christie Phase 1 and two with Phase 2. The results are recorded in Fig. 4*a* and *b*. The serological response observed in these two groups of rabbits is almost similar to that obtained in rabbits vaccinated with Nine Mile Phase 1. When the animals were reinoculated with the same antigens 125 days after the first injection there was a sharp rise in all the antibody titers.

Discussion

The results reported above indicate that the Phase 1 and Phase 2 variants of *R. burneti* differ in their antigenic properties. The differences, however, are more striking between the variants of the Nine Mile strain than between those of the Christie strain.

When animals are infected or vaccinated (killed suspensions) with the Phase 1 variant of the Nine Mile strain it is found that Phase 2 antibodies (Nine Mile and Christie) appear very early after inoculation whereas Phase 1 antibodies (Nine Mile and Christie) become detectable much later. This seems to indicate that the Phase 1 variant of Nine Mile contains two types of antigenic components: one with Phase 1 specificity and the other with Phase 2. Let us call these components 1 and 2 respectively. The inability of Phase 1 organisms to react with the Phase 2 antibodies they have elicited, which are detectable in early antisera, seems to indicate that component 2 in the Phase 1 organisms is unavailable for union with antibody *in vitro*. In other words, it would appear that component 2 is masked by component 1

which behaves as a surface antigen. Components 1 and 2 are probably of a different chemical composition since the stimulus they offer to the antibody-producing mechanisms is different; antibodies against component 2 appear much earlier than those against component 1. One would be tempted to call component 2 a "good" antigen and component 1 a "poor" antigen.

When guinea pigs are infected with the Nine Mile Phase 2 strain, the serological response is not very different from that observed in animals infected with the Nine Mile Phase 1 variant. It will be recalled that Stoker and Fiset (14) have shown that Phase 2 organisms revert readily to Phase 1 by passage in guinea pigs. This may explain why only minor differences are observed between the two groups of animals.

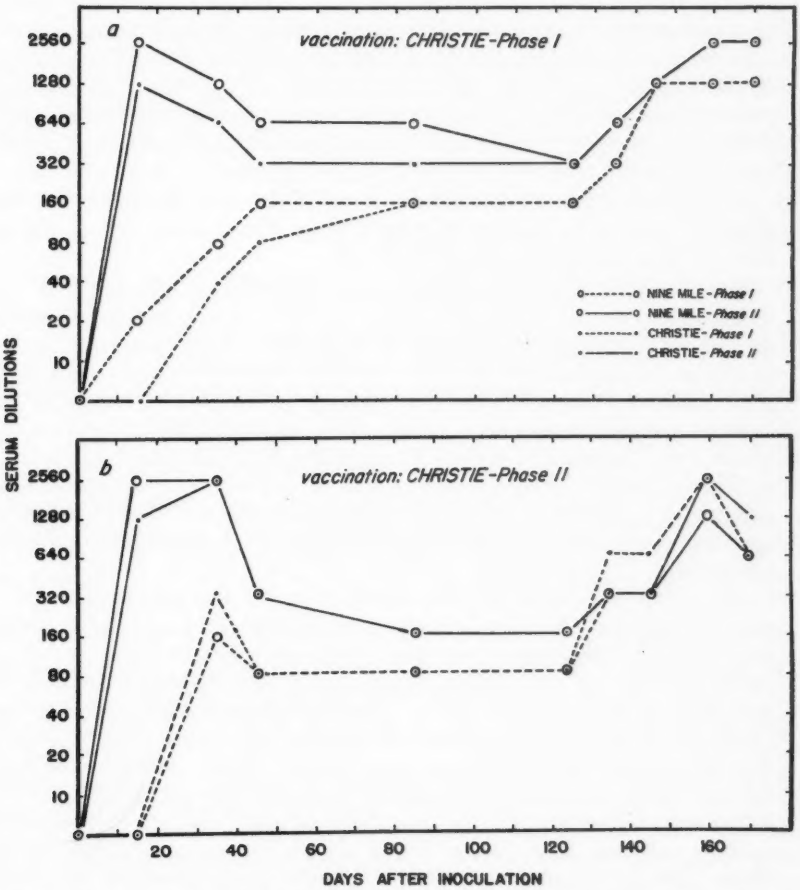

FIG. 4. Antibody response in rabbits vaccinated with killed suspensions of (*a*) Christie, Phase 1, and (*b*) Christie, Phase 2.

When guinea pigs are vaccinated with a killed purified suspension of the Phase 2 variant of Nine Mile they develop only anti-Nine Mile Phase 2 antibodies. This would indicate that the Nine Mile strain loses the surface component 1 when it reverts to Phase 2 in the course of egg adaptation. It would also seem that in the process of reverting to Phase 2 it loses the components responsible for its cross-reactions with the Christie strain. That the component 1 (Nine Mile) and the components responsible for Christie activity do not disappear completely from the Nine Mile Phase 2 population is indicated by the production of anti-Phase 1 antibodies (Nine Mile and Christie) and Christie Phase 2 antibodies in vaccinated rabbits, particularly after a late booster injection.

The study of the antibody response to the Christie strain was limited to vaccination, with killed suspensions, of guinea pigs and rabbits. The results indicate that the Phase 1 and Phase 2 variants of this strain differ very little in their ability to induce antibody production. Both strains seem to have components 1 and 2, and the serological response they stimulate is similar to that obtained with the Nine Mile Phase 1 variant. They differ, nevertheless, in their ability to combine with antibody *in vitro*.

The Christie Phase 1 variant seems to differ very little from the Nine Mile Phase 1; both fail to react with early antisera (Christie and Nine Mile) and both stimulate a similar antibody response.

The Christie Phase 2 and the Nine Mile Phase 2, though similar in their ability to combine with antibody *in vitro*, stimulate different antibody responses in animals. Whereas killed suspensions of Nine Mile Phase 2 induce the production of antibodies that react only with Nine Mile Phase 2 (in guinea pigs), Christie Phase 2 induces the production of antibodies that react with both phases of both strains. It must be remembered that the Nine Mile Phase 2 variant has had almost 60 egg passages and is thoroughly adapted to this host, while the Christie Phase 2 strain has had only nine egg passages and has only recently changed to Phase 2. It may be that the rickettsial population of Christie Phase 2 still contains a large proportion of Phase 1 particles.

The evidence presented might explain some of the results obtained by previous workers. Robbins *et al.* (10) studied several strains of *R. burneti* isolated during the outbreaks of Q fever that occurred among United States troops stationed in the Mediterranean area during the winter of 1944 and the spring of 1945. These "Italian" strains were compared with an American (Dyer) strain and an Australian strain isolated by Burnet and Freeman (1). The Italian and Australian strains reacted readily in the complement fixation test with convalescent human serum whereas the Dyer strain failed to react unless very concentrated suspensions were used. Guinea pigs, whether infected with the Dyer strain or one of the Italian strains (Henzerling), showed identical antibody responses. Antibodies against the Henzerling strain could be detected by the 15th day after infection while antibodies against the Dyer strain were not detectable before the 40th day. The Dyer

antibodies eventually reached the same titers as the Henzerling. Smadel *et al.* (11) carried out similar experiments in guinea pigs with killed purified suspensions of the Henzerling and Dyer strains. The animals produced antibodies first against the Henzerling strain and later against the Dyer. These results were not different from those reported previously by Robbins *et al.* (10). It would seem from the results just quoted that the Henzerling and Australian strains were in Phase 2 while the Dyer was in Phase 1. However, the results of Smadel *et al.* (11) seem to indicate that the Henzerling strain still contained a large proportion of Phase 1 particles, since the response to vaccination resembles that obtained with Christie Phase 2.

Acknowledgments

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BACTERIA FOUND IN THE AIR OVER CANADA AND THE AMERICAN ARCTIC[1]

C. D. KELLY AND SHARON LAYNE

Abstract

The bacteria isolated from the air in a series of aeroplane flights, over Canada, Alaska, and the Arctic Ocean, are discussed. All bacteria are forms commonly found in the soil and include species of *Flavobacterium, Achromobacterium, Pseudomonas, Corynebacterium, Micrococcus, Sarcina,* and *Bacillus.* An attempt is made to correlate the types of organisms found with the air masses. Air that had received no recontamination for some time tended to show death of organisms in a certain order. The first to be reduced in numbers were the Gram-negative rods, then the Gram-positive rods, followed by the micrococci, and finally the sporeformers.

Introduction

During the summer of 1947 agar plates were exposed from aircraft, by hand, over the Canadian Arctic (18, 15). On incubation a number of fungi and a greater number of bacteria were found growing on the plates. Studies were continued on the microflora of arctic air to determine possible differences between these organisms and those found in non-arctic air (12). As a basis of comparison and to study the bacteria and fungi in various types of air masses, samples were taken over the non-arctic region of Canada (6, 7) as well as over the Atlantic Ocean (13, 14).

From the time Nystrom sampled air for bacteria at Spitzbergen in 1868 several attempts have been made to study bacteria in arctic air (5). While the data up to 1947 were meager the indications were that arctic air contained very few microorganisms. Determinations over the arctic from 1947 to 1950 demonstrated the presence of bacteria but they were few in numbers (12, 17). Studies in the antarctic reported even fewer bacteria than in the arctic (3). Investigations in temperate zones have been more extensive and Proctor (19) and Wolfe (20), in bacterial studies over land, report finding Gram-negative rods, micrococci, sarcinae, and bacilli, which they point out were soil forms. Zobell and Mathews (21), in studies over the sea, found marine forms.

The bacteria reported here were collected on the following flights, viz. (i) from Fairbanks, Alaska, to the North Pole (17); (ii) from Winnipeg, Manitoba, to Baker Lake, N.W.T., and back to Churchill, Manitoba; (iii) return flights between Churchill and Resolute, N.W.T. (12); (iv) return flights between Montreal and Whitehorse, Yukon, under summer and winter

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conditions; (v) Montreal to Edmonton; (vi) Fairbanks and Edmonton (6). All flights were at an altitude between five thousand and ten thousand feet. Samples were also taken on top of the hangar at the Churchill airport.

The flights to the North Pole were carried out with the aid and facilities provided by the United States Air Force. All other flights were made by the aid of the Royal Canadian Air Force, which has helped greatly in these investigations.

Following the first studies of 1947, quantitative sampling methods were developed and after the autumn of 1948 all samples were reported on a cubic foot basis. The samples were taken in glass wool filters and onto Petri plates with a "G.E. Electrostatic Bacterial Air Sampler" housed in a metal box in the body of the aircraft. Air from the exterior of the aircraft was led into and out of the box, through elbows in the window of the aircraft and through 1-in. hose pipes. This equipment was known as the "McGill G.E. Sampler" (8). The medium used was Czapek's solution agar modified by omitting the sucrose and adding 0.1% yeast extract (18). This was a fortunate choice of medium as both the fungi and bacteria grew readily. At times, particularly during winter, it was found that the agar would freeze on exposure to low temperatures and under such conditions the agar was replaced by smearing silicone grease over the bottom of the Petri plate to catch the microorganisms (11). Following sampling, agar was poured over the silicone in the plates before incubation at room temperature for 5 days.

Whenever possible, all the bacterial colonies on a plate were fished off for further study. But in cultures in which large numbers of colonies developed only the colonies on a section of a plate were isolated for study. For further studies after isolation, nutrient agar was used and the bacteria classified according to Bergey's Manual (1).

Summary of Flights

Flights over the Canadian Arctic between August 12 and September 5, 1947 (15)

As the 52 samples were qualitative little can be said about numbers and as the bacteria were not identified beyond main groups, they were recorded as numbers isolated and percentages of each group (Table I). Exposures were for 2 minutes and bacterial counts varied from 14 to 530 per plate.

Flights between Fairbanks and the North Pole September 13, 1948, and March 28 and 29, 1949 (17)

These three flights were made from Fairbanks, Alaska, north over the arctic ice in a B29 aircraft by facilities provided by the Alaska Command of the United States Air Force. Two of these flights, September 13, 1948, and March 28, 1949, were to the North Pole but on the third flight, March 28, 1949, the aircraft was obliged to turn back at 75° N. Details of these flights were published by Polunin in 1951 (17).

On the September flight, qualitative samples were taken by exposing Petri dishes in front of the nose of the aircraft with a specially designed apparatus

TABLE I

TYPES OF BACTERIA FOUND IN ARCTIC AIR

Flight	Gram-negative rods		Gram-positive rods		Micrococci and Sarcina		Aerobic sporeformers	
	No.	%	No.	%	No.	%	No.	%
Northern Canada, Aug. 1947	32	20.8	66	42.8	48	31.2	8	5.2
North Pole, Sept. 1948	30	22.9	42	32.0	33	25.2	26	19.9
North Pole, Mar. 1949	21	9.7	21	9.7	20	9.3	153	71.3
Winnipeg to Baker Lake and Churchill, Aug. 1949	3	7.3	7	17.1	20	29.2	11	26.8
Local flight Churchill, Aug. 1949	1	4.2	14	58.3	7	29.2	2	8.3
Churchill to Resolute and return, Aug. 1950	0	0.0	10	23.8	10	23.8	22	52.2
On hangar at Churchill, Aug. 1950	215	20.0	502	46.8	157	14.6	199	18.6

(8). On the two flights, March 28 and 29, quantitative samples were taken with the McGill G.E. sampler onto silicone coated Petri dishes and in glass wool filters (8).

The results of these flights could not be compared directly as the September samples (at the end of summer) were qualitative and the March samples (at the end of winter) were quantitative. However the percentages of the main groups of bacteria could be compared. The results of all three flights showed that this air, both in summer and winter, was nearly sterile.

Flights, September 1948

The September flight was through air masses that had moved from northern Asia and plates exposed for 1, 3, 5, and 10 minutes to the air gave numbers that varied from 0 to 28 bacterial colonies per plate. From the plates, 131 pure cultures of bacteria were isolated and classified into genera and in most cases to species according to Bergey's Manual (1).

Eighteen of the cultures were found to belong to the genus *Flavobacterium*. Five were *F. aurantiacum:* four *F. aquatile*, two *F. rhenanus*, and one each of *F. fucatum*, *F. suaveolens*, *F. lutescens*, *F. buccalis*, and *F. matzoonii*. Eight cultures were placed in the genus *Achromobacter* with two as *A. solitarium*, two *A. dendriticum*, one each of *A. candicans*, *A. pikowskyi*, and *A. liquidum*, and one that could not be classified beyond the genus. Four of the isolates were *Pseudomonas*, three being *P. ambigua* and one *P. nebulosa*.

Forty-two of the cultures isolated were Gram-positive pleomorphic rods which are representative of a large group of organisms so common in soil and which have presented so much of a problem as to their classification.

The strains that could be classified are placed by Bergey's Manual (1) in the genus *Corynebacterium* following Jensen. Though 15 of these strains could not be completely classified they are placed in the genus *Corynebacterium* for the time being at least. Of the remaining 27 strains, seven were *C. equi*,[2] seven *C. tumescens*, seven *C. simplex*, four *C. helvolum*, and two *C. filamentosum*.

The 16 micrococci found were classified into four strains of *Micrococcus aurantiacus*, three *M. candidus*, two *M. flavus*, two *M. rubens*, and one each of *M. liquefaciens*, *M. conglomeratus*, *M. pyogenes*, *M. roseus*, *M. rhodochrous*. Sarcinae were represented by 17 strains of which eight were *Sarcina* that could not be classified as to species. The remaining strains were three *S. flava*, *S. lutea*, and one each of *S. aurantiaca*, *S. subflava*, *S. hamaguchii*, *S. rhodochrous*.

Out of the 26 strains of aerobic sporeformers only one could not be classified. The remaining 25 strains were five *Bacillus subtilis*, four *B. laterosporus*, three each of *B. pumilus*, *B. megaterium*, *B. cereus*, two *B. firmus*, and one each of *B. mycoides* and *B. brevis*.

Flight, March 1949

These two flights were carried out over the Arctic Ocean to determine what organisms were carried in the air at the end of winter, as compared with the end of summer. While spring had started in the Fairbanks area, winter conditions prevailed north of the Brooks Range. The air sampled had moved from northern Asia where winter conditions also prevailed.

The numbers of organisms found were so low that the air could be considered almost sterile. The greatest number of bacterial colonies on a single plate after the sampling of 60 cu. ft. of air was six and the counts ranged from 0.06 to 0.33 bacteria per cubic foot. In all, 215 cultures were isolated and classified as far as possible.

Twenty-one colonies were Gram-negative rods, 10 of which could not be classified further, eight were *Pseudomonas*, and three were *Achromobacter*.

Twenty-one colonies of the genus *Corynebacterium* were found on the plates, but 10 did not grow on subculture and two could not be classified beyond the genus. Of those that could be classified four were *C. equi*, three *C. simplex*, and one each was *C. tumescens* and *C. helvolum*.

Twenty cultures of *Micrococcus* were isolated, 12 of which could not be classified as to species. Two cultured were *M. flavus*, two *M. conglomeratus* with one each of *M. caseolyticus*, *M. varians*, *M. luteus*, and *M. albus*. No *Sarcina* were found.

The remaining 153 of the 215 cultures were isolated aerobic sporeformers, 34 of which, though they belong in the genus *Bacillus*, could not be classified as to species. Of the remainder, 45 were *B. brevis*, 30 *B. macerans*, 22 *B. circulans*, 15 *B. subtilis*, six *B. laterosporus*, and one *B. pumilus*.

[2] Organisms that fit the description of *C. equi* have been found abundantly in soil by Jensen (4).

Winnipeg to Baker Lake and back to Churchill, July 19, 1949 (12)

This flight, from Winnipeg to Baker Lake and back to Churchill, was all in fresh arctic air that had moved down, the previous day, from north of Hudson Bay. The bacterial counts from the McGill G.E. samples were low and with the exception of one (1.1 per cu. ft.) were all between 0.3 and 0.1 per cu. ft. (12).

Because of an unfortunate occurrence the Gram-negative rods and the sporeformers were lost. Though the record is incomplete the Gram-positive rods and micrococci are reported.

Ten cultures of Gram-positive pleomorphic rods were identified as one *Corynebacterium equi*, five *C. simplex*, two *C. tumescens*, and two *C. helvolum*. There were 22 strains of *Micrococcus*, six of which were *M. flavus*, three *M. luteus*, five *M. casolyticus*, three *M. conglomeratus*, with one each of *M. citreus*, *M. candidus*, *M. albus*, *M. rhodochrous*, and *M. freudenreichii*. Two cultures were identified as *Sarcina flava* and two as *S. lutea*.

Two Return Flights from Churchill to Resolute Bay, 1950 (12)

On August 1, 1950, a flight was made from Churchill to Resolute, with the return on the following day by way of Coral Harbor on Southampton Island (12). The flight was repeated on August 3 and throughout the two flights the air sampled was all of arctic origin which had come from the north of Hudson Bay. The numbers of bacteria were low and with the exception of one sample (1.07 bacteria per cu. ft.) were all between 0.04 and 0.67 per cu. ft.

The bacteria isolated were all similar to those found on previous flights. One each of members of the genus *Flavobacterium* and *Achromobacter* were found but did not fit any known species. Two members of the genus *Pseudomonas* were also found and could not be carried further. Six cultures of *Corynebacterium* were isolated and four were further classified as two each of *C. simplex* and *C. tumescens*. Of the 11 cultures of *Micrococcus* species, six were *M. caseolyticus*, three *M. luteus*, and one each of *M. conglomeratus* and *M. rhodochrous*. A single culture of *Sarcina flava* was found. The aerobic sporeforming rods were the group most common in this air and were represented by six strains of *B. subtilis* and nine of *B. megaterium*.

Churchill at Ground Level (12)

From the bacterial species found on these arctic flights it seems clear that the flora of Arctic air is little different from that of air in more temperate zones and that the bacteria are the common soil forms. This has been well established for temperate zones by a number of investigators and particularly Proctor (19) and Wolfe (20). Because of these commonly occurring species it did not seem desirable to classify fully all the strains isolated, and more information could be collected by classifying the bacteria into main groups on morphological grounds. Following this procedure, the bacteria found on plates and in filters from the three sets of samples taken on the Baker Lake and the local Churchill flights, as well as those taken on the roof of the hangar

at Churchill, were stained by the Gram method and divided into four groups on their morphology. These groups are Gram-negative rods, Gram-positive non-sporeforming rods, cocci in clusters, and sporeformers. The group of Gram-negative rods is made up mainly of the genera *Flavobacterium*, *Achromobacterium*, and *Pseudomonas*. The Gram-positive non-sporeforming rods are mainly those soil forms that have been placed, for the present, in the genus *Corynebacterium*. *Sarcina* and *Micrococcus* make up the cocci in clusters and the sporeformers include only the genus *Bacillus*. Of 1073 colonies examined 215 were Gram-negative rods, 502 Gram-positive non-sporing rods, 157 micrococci, and 199 sporeformers (Table I).

Flights from Montreal to Whitehorse and back during March 7, 8, 9, 1949 (6)

The bacteria isolated on the flights to Whitehorse are classified in the text. The percentages of the bacterial groups found on the Whitehorse flights (one to Edmonton and one from Fairbanks to Edmonton) are given in Table II. Included are the percentages of bacteria found on two single flights (one from Montreal to Edmonton and one from Fairbanks to Edmonton).

On this flight the air encountered was all from the north. During the first two days the air entered Canada from the Pacific Ocean by the way of Alaska and was designated "polar pacific". On the last day the air mass was "continental polar" since it came from Northern Canada. All samples were low in count and none were over one per cubic foot.

TABLE II

BACTERIA FOUND IN THE AIR OF MORE TEMPERATE PARTS OF CANADA

Date	Sample	Air mass*	No. bacteria examined	Percentage of bacterial groups			
				Gr-neg. rods	Gr-pos. rods	Micro.	Sp. formers
Mar. 7, 1949	Montreal to Edmonton	p^P	136	4.9	35.7	21.7	37.7
Mar. 8, 1949	Edmonton to Whitehorse and back	p^P	196	20.8	17.4	45.0	16.8
Mar. 9, 1949	Edmonton to Montreal	c^P	94	21.3	40.4	7.4	30.8
Mar. 17, 1949	Montreal to Edmonton	c^P	143	13.2	28.0	17.3	41.5
Mar. 30, 1949	Fairbanks to Edmonton	p^P	65	3.7	26.0	21.3	49.0
Aug. 22, 1949	Montreal to Edmonton	c^P	53	7.6	39.6	28.3	24.5
Aug. 22, 1949	Montreal to Edmonton	mc^P	76	11.8	51.2	27.6	9.4
Aug. 23, 1949	Edmonton to Whitehorse and back	p^P	112	17.0	27.2	23.9	31.9
Aug. 24, 1949	Edmonton to Montreal	p^P	27	14.8	29.6	7.4	48.9
Aug. 24, 1949	Edmonton to Montreal	c^P	42	7.1	35.7	26.2	31.0
Aug. 24, 1949	Edmonton to Montreal	mc^P	83	3.6	36.2	2.4	57.8
Oct. 22, 1948	Edmonton to Whitehorse and back	p^P	104	10.8	16.3	60.4	12.5

NOTE: p^P, Pacific polar air; c^P, continental polar air; mc^P, modified continental polar air.

During this flight collections were made from which the following cultures were isolated and studied. Only one culture of Gram-negative rods was isolated; its identification was carried only to the genus, which was *Alkaligenes*.

Of the Gram-positive rods, eight were classified as: three strains of *Corynebacterium tumescens*, two each of *C. equi* and *C. simplex*, and one *C. helvolum*.

Eleven strains were in the genus *Micrococcus* and were classified as: five strains of *M. flavus*, two *M. coagulans*, two *M. caseolyticus*, *M. luteus*, one *M. candidus*, and one *M. citreus*.

Of the sporeformers, only one of each of the following four species were represented: *Bacillus cereus* var. *mycoides*, *B. coagulans*, *B. pumilus*, and *B. cereus*.

Flight from Montreal to Whitehorse and back during August 22, 23, 24, 1949

Three air masses were encountered during this 3 day flight. On the first day from Montreal to Edmonton "mixed continental polar" air was encountered in the east and "continental polar" in the west. On the second day the entire flight from Edmonton to Whitehorse and back was through a "polar pacific" air mass. On the final day the flight back to Montreal was through "polar pacific" air for a short time after leaving Edmonton, then "continental polar" air, and finally "mixed continental polar" air in the east. All the counts were less than one bacterium per cubic foot of air, except in a sample that showed 1.0 per cu. ft., and one that had 2.3 per cu. foot.

Some 55 cultures were isolated and classified as far as possible. Eight Gram-negative rods on isolation were placed in the genus *Pseudomonas* but could not be classified further. Twenty-one species were placed in the genus *Corynebacterium* and classified as to species. Eight strains were *C. equi*, four were *C. tumescens*, three *C. helvolum*, six *C. simplex*, and one *C. agropyri*. Thirteen species were of the genus *Micrococcus*. Four were *M. flavus*, two *M. candidus*, two *M. freudenreichii*, and one each of *M. citreus* and *M. rubens*. Included in this group are two strains of *Sarcina flava* and one of *S. aurantiaca*. Eight strains of *B. subtilus* were isolated, two of *B. alvei* and one each of *B. cereus* and *B. pumilus*.

Discussion

In their inception these aerobiological studies were concerned with the numbers and species of bacteria and fungi in the arctic air, but particularly with determining whether there was a distinct flora of arctic microorganisms. The fungi have been examined and discussed by Pady, Peterson, and Green (16), and Pady and Kapica (10), who concluded that few if any of the fungi found in arctic air were of arctic origin.

From an examination of the bacteria, the species found and identified were similar to those found by Proctor (19), Wolfe (20), and others in air over non-arctic regions. In studies of bacteria in arctic soil (5), the general conclusion has been that they are not different from those found in soil the world over, though the numbers are much reduced. In much of the Canadian

Arctic, the "perma-frost" lies but a few inches below the surface, while the surface soil, when not frozen, is saturated with water. There is little opportunity for bacterial growth in this soil and because of the water-saturated condition very few bacteria could be carried into the air. Any of these arctic soil bacteria that might be carried into the air would be difficult to distinguish from those that had been carried from other regions. Therefore there is little doubt that the bacteria found in arctic air were of non-arctic origin but had come from agriculture land farther south.

Gram-positive pleomorphic non-sporing rods were found in all air-masses sampled and at times represented a fairly high percentage of the organisms isolated. This, at first, seemed surprising as they had not been reported in previous studies of bacteria in the air. It should not be surprising, however, to find these Gram-positive rods in air since they constitute quite a high percentage of the flora of soil (2). It would seem that Czapek's agar was a much more suitable medium for growing these organisms than the agar used by both Proctor (19) and Wolfe (20). Lochhead points out that beef peptone agar is not at all satisfactory for growing this group of organisms (9).

As mentioned earlier in the ground level studies at Churchill, the bacteria were separated on morphological grounds into four groups as Gram-negative rods, Gram-positive pleomorphic rods, micrococci, and sporeforming rods. With this division of the bacteria much more material could be examined with results that were equally satisfactory. The particular species found in any one genus appears to be but a matter of chance and therefore would give little more information than would identification of the group or genus. Lochhead (9), in studies of soil bacteria, identified them on morphological grounds but divided them into eight groups. It was considered, after identifying species on a number of flights, that for these studies in aerobiology, four groups would give the necessary information.

The Gram-negative rods include *Flavobacterium*, *Chromobacterium*, and *Pseudomonas*. The Gram-positive pleomorphic rods are made up largely of a group that for the time being are placed in the genus *Corynebacterium*, but also include the *Bacterium* (*Arthrobacter*) *globiforme* group.

It seems to be well established that the bacteria of air are of soil origin and get into the air in greatest numbers during spring and autumn, at the period of greatest cultivation (6). As there is no growth of bacteria in the air, recently infected air should carry a representative flora as found in soil, but much reduced in numbers. Washing by rain would take out many bacteria but should not change the ratio of the various soil types. In time, with no fresh contamination, there would be a reduction in numbers owing to death from exposure. The more susceptible types would die out first and the ratio of groups would be changed. Therefore, the ratio of bacterial groups in air should give some indication of how recently there has been infection.

It is not easy to establish a definite ratio of these groups in soil because of variation in numbers with the seasons of the year and soil conditions.

Lochhead (9) in the course of some soil studies found that these morphological groups varied but did show a ratio within certain limits. The Gram-negative rods were present in 13.3 to 30.5%, Gram-positive rods 37.0 to 71.1%, cocci 0.0 to 7.7%, and sporeformers 4.2 to 10.0%. It would be expected that air freshly inoculated from soil would have approximately this same ratio of organisms. The percentages of the various groups found on the flights in 1947, as well as on the local flight at Churchill in 1948, come close to the ratio for soil (Tables I and II) and could be considered as air that had been over agricultural land and that had received a fresh inoculation of bacteria not too long before. On the other hand, air sampled on the North Pole flights of March 1949 and that examined on flights to Resolute during 1950 showed a low percentage of Gram-positive and Gram-negative rods and higher percentage of sporeformers, and cocci, which indicated that it had been a considerable time without fresh contamination. Samples taken on the 1948 North Pole flight and the Baker Lake flight and the ground level samples showed an intermediate distribution of organisms (Table I).

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BACTERIA FOUND IN THE AIR OVER THE ATLANTIC OCEAN[1]

C. D. Kelly and Sharon Layne

Abstract

Two return flights were made across the Atlantic Ocean from Montreal to London, England, and aerobiological samples were taken at intervals throughout the flights. This publication deals with the bacteria isolated during these flights. The bacteria were found to be very much like those found over land and included the following genera: *Pseudomonas*, *Flavobacterium*, *Achromobacter*, *Corynebacterium*, *Micrococcus*, *Sarcina*, and *Bacillus*.

Since 1947 four aerobiological studies have been carried out on the microorganisms in arctic and non-arctic parts of Canada as well as in Alaska and over the Arctic Ocean. The types of fungi (5) and bacteria (2) found were apparently of soil origin and many forms remained suspended in the air for a considerable time. The results of examination of various types of air at different periods of the year indicate that in time the microorganisms died in a certain order and the air eventually became sterile. Air that had been over the arctic for a considerable time, as well as winter air, carried small numbers of the more resistant types of microorganisms. The fungi in such air were few in numbers or not present, indicating that they had a lower resistance than the bacteria. The sporeformers showed the greatest resistance and at times were the only organisms present. Because of possible recontamination of the air from soil, it was difficult at times to determine with certainty which organisms had survived and which had been more recently swept into the air. Though Zobel has shown (8) that bacteria from the ocean may be found in maritime air, sampling from the air over the ocean seemed to present the best possibility of determining survival types. Antarctica, since it is far removed from arable land by wide stretches of water, would seem to be an ideal place for such sampling. However, Darling and Siple (1) found the air, at ground level, to be practically sterile, with sporeforming bacteria and *Achromobacter* species the few remaining organisms.

It seemed desirable to collect samples over the ocean, and therefore two sampling flights, over the Atlantic Ocean between Montreal and London, England, were carried out in June and August 1951 (6). The June flight was by way of Goose Bay to London and from London to Iceland, to Greenwood in Nova Scotia, and back to Montreal. The August flight was by way of Goose Bay to London and the return to Montreal, by way of Iceland and Goose Bay. Flights were at eight and nine thousand feet. All air masses were recorded and later correlated with the samples.

The technical data of the flight itself was recorded earlier (6) and will not be repeated in detail here. Samples were taken by the McGill G.E. sampler (2) onto Petri dishes containing modified Czapek's agar and with air filters

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TABLE I

TYPES OF BACTERIA IN AIR MASSES OVER THE ATLANTIC OCEAN

Flight	Air masses	Gram-negative rods		Gram-positive rods		Micrococci		Sporeformers	
		No.	%	No.	%	No.	%	No.	%
June	Tropical	8	6.5	24	19.5	36	29.2	55	44.7
	Polar	2	1.8	13	11.6	78	69.2	19	17.0
August	Tropical	17	18.1	44	46.9	13	13.8	20	21.2
	Polar	17	19.7	20	23.3	11	12.8	38	44.2

which were later plated on Czapek's agar. The samples were incubated for 5 days at room temperature. All colonies were isolated and studied. The cultures were divided into groups of Gram-negative rods, Gram-positive pleomorphic rods, staphylococci, and sporeforming rods, and the percentage of each group of organisms are reported in Table I. The bacteria were also classified into genera and species and recorded in the results.

Results

The numbers of bacteria in the Atlantic air were very low and counts per cubic foot varied from 0.0 to 0.5 in polar air masses and 0.3 to 0.9 in tropic air, so it was difficult to estimate percentages of bacterial species from such low numbers. The fungus counts were somewhat higher, being 0.05 to 2.6 per cu. ft. in polar air and 0.2 to 9.0 in tropical air. Somewhat similar counts were found in air over non-arctic Canada in summer time (3).

Pady and Kapica reporting on the fungi over the Atlantic (4) considered that the number of non-viable fungus spores on silicon slides, as compared with the number of viable spores on agar plates, gave a good indication of the history of an air mass or the length of time since the air had picked up spores by passing over cultivated land. As there was no method available for estimating non-living bacteria in the air, it was not possible to make such a comparison with the bacteria. However, the percentage of certain types of bacteria does give some indication of the history of air masses. The percentages of the different types for polar and tropical air are given in Table I. There were very few *Sarcina* found on the plates excepting on one plate which contained 49 *Sarcina* from tropical air and no other organisms. This plate was eliminated from the averages, but some of the organisms have been identified.

The species of bacteria were the same as found in air masses over land but were inclined to be fewer in numbers. The numbers in tropical air were higher than in polar air and there was a difference in the ratio of types (Table I).

June Flights

Two hundred and four organisms were isolated and identified as completely as possible: five were species of *Pseudomonas* but could not be identified further; two were species of *Flavobacterium* (*F. breve* and *F. arborescens*); three cultures of *Achromobacter liquefaciens* were found. One Gram-negative rod was lost before it could be identified.

Thirty-eight species of Gram-positive pleomorphic rods were all placed in the genus *Corynebacterium*. Eleven were *C. simplex*, nine were *C. helvolum*, and six were *C. tumescens*. Twelve could not be identified as to species but could be taken only as far as the genus.

One hundred and four cultures were placed in the genus *Micrococcus*, and 13 species of *Sarcina* were isolated and the species determined where possible. Ten were classified as *M. saccatus*, seven as *M. freudenreichii*, 13 as *M. ureae*, one as *M. nitrificans*, four as *M. flavus*, 13 as *M. candidus*, nine as *M. varians*, seven as *M. luteus*, six as *M. epidermidus*, two as *M. auranticus*, one as *M. albus*, and four could not be carried farther than the genus. The *Sarcina* were three *S. lutea*, one *S. aurantiaca*, one *S. lactea*, two *S. subflava*, one *S. conjunctivae*, and four were unclassified as to species. The genus *Bacillus* was represented by 42 species. The species were 15 *B. brevis*, 30 *B. cereus*, 10 *B. megaterium*, two *B. macerans*, six *B. subtilis*, one *B. polymyxa*, and eight species which could not be identified.

August Flights

On these flights 180 organisms were isolated and classified. The Gram-negative rods were represented by a total of 35 strains of which *Achromobacter eurydice* was represented by five strains and *Flavobacterium marinum* by one strain. Twenty-seven were strains of *Pseudomonas* that could not be classified beyond the genus and two could only be classified as Gram-negative rods.

The Gram-positive pleomorphic rods were represented by 65 species and were broken down as 20 species of *Corynebacterium simplex*, 17 as *C. helvolum*, one *C. fimi*, two *C. tumescens*, one *C. filamentosum*, and 24 unclassified *Corynebacteria*.

The 24 micrococci were classified as four *M. caseolyticus*, two as *M. candidus*, seven as *M. epidermidus*, one *M. luteus*, one *M. flavus*, two *M. ureae*, one *M. saccatus*, one *M. aurantiacus*, three *M. varians*, one *M. albus*, and one unclassified.

Discussion and Conclusions

At eight thousand to nine thousand feet the number of bacteria found over the ocean was much the same as found over land, over the ocean there was less chance, however, that there had been fresh inoculation. The bacteria isolated on both flights were common soil types that could be found by sampling soil at random.

After these bacteria from over the Atlantic Ocean were isolated and classified, it was concluded that they were very similar to those found over land. It was also felt that groups of similar organisms would give as much information

as would the species completely classified. Therefore, in Table I the bacteria are reported as Gram-negative rods, Gram-positive pleomorphic rods, Gram-positive pleomorphic rods, micrococci, and sporeformers.

The ratio of the types of bacteria could be used to some extent to trace the history of an air mass. The tropical air tended to show a higher percentage of Gram-negative rods and Gram-positive pleomorphic rods than micrococci and sporeformers, while arctic air tended to show the opposite.

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THE CYSTEINE DESULPHYDRASE OF PSEUDOMONAS SALINARIA[1]

R. M. BAXTER AND N. E. GIBBONS

Abstract

Cells of *Pseudomonas salinaria* contain a cysteine desulphydrase which, like the corresponding enzyme of *Escherichia coli*, decomposes cysteine to hydrogen sulphide, pyruvic acid, and ammonia in the presence of pyridoxal phosphate. Unlike the enzyme of *E. coli*, the enzyme of *P. salinaria* requires high concentrations of potassium chloride for maximum activity. The enzyme is present in cells grown in media containing 15 to 30% sodium chloride.

Introduction

A characteristic widely used in bacterial taxonomy is the production of hydrogen sulphide by growing cultures. In most heterotrophic bacteria the enzyme responsible is cysteine desulphydrase, which is thought to decompose cysteine to hydrogen sulphide and an unstable intermediate which hydrolyzes spontaneously (6); the final products are hydrogen sulphide, pyruvic acid, and ammonia.

Pseudomonas salinaria and certain other red halophiles produce hydrogen sulphide during growth in media containing 25 or 30% sodium chloride, but not in media containing 15 or 20% (7). It was of interest, therefore, to determine whether *P. salinaria* produced hydrogen sulphide by a mechanism similar to that of other heterotrophs, and whether the failure of the organism to produce hydrogen sulphide at the lower salt concentrations was due to failure to produce the enzyme or to some other cause.

Materials and Methods

Procedures for the growth of *P. salinaria* and *Escherichia coli* and preparation of cell-free extracts have been described in previous papers (2, 3). The medium for *P. salinaria* contained 25% sodium chloride (w/v) unless otherwise stated. Extracts for experiments in which ammonia was measured were dialyzed overnight in the cold against saturated potassium chloride solutions.

A stock solution of 0.5 *M* cysteine hydrochloride was prepared and stored in the cold. Just before use an appropriate volume of this solution was mixed with an equal volume of 1.0 *M* sodium phosphate, pH 7.2.

Warburg flasks with two sidearms were used as reaction vessels. The main compartment contained the bacterial cell suspension or extract, 0.1 or 0.2 ml. of sodium phosphate buffer (1.0 *M*, pH 7.2), and other additions to give a total volume of 3.0 ml. One sidearm contained 0.2 ml. of cysteine solution, and the other contained 0.1 ml. of 10 *N* sulphuric acid. The center

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well contained a small roll of filter paper and 0.2 ml. of zinc acetate solution (20 g. zinc acetate dihydrate per 100 ml.). The flasks were attached to manometers, placed in a water bath at 29.5° C., and flushed with nitrogen. The reaction was started by tipping in the cysteine and stopped by tipping in the acid. About 15 minutes after the acid was added, the units were dismantled and the filter paper rolls and center well washings placed in 50 ml. volumetric flasks. The sulphide was converted to methylene blue by oxidation with ferric chloride in the presence of *p*-dimethylaminoaniline in acid medium (1). The optical density of the resulting dye was measured at 660 mμ using a Coleman Junior spectrophotometer and the amount of hydrogen sulphide originally present was determined by reference to a standard curve prepared using a solution of sodium sulphide, standardized iodimetrically. When pyruvate was to be determined, the flask contents were centrifuged and pyruvic acid estimated on aliquots of the supernatant as the 2,4-dinitrophenylhydrazone (5). Ammonia was determined on parallel sets of flasks with saturated potassium carbonate solution in the second sidearms and 1.0 *N* sulphuric acid in the center wells. The ammonia was distilled from the reaction mixture (4) and estimated by the use of Nessler's reagent.

Results

Hydrogen sulphide, pyruvic acid, and ammonia were produced during incubation of *P. salinaria* extract with cysteine (Table I). Approximately equimolar quantities of hydrogen sulphide and pyruvic acid were formed and although yields of ammonia were variable, there seems no reason to doubt that *P. salinaria*, like many organisms, contains a cysteine desulphydrase which breaks down cysteine to yield equimolar amounts of hydrogen sulphide, pyruvic acid, and ammonia. Very little hydrogen sulphide or pyruvic acid was formed in the absence of added cysteine, but endogenous ammonia production was high, even with dialyzed extracts. This may account in part for the variable yields of ammonia.

TABLE I

PRODUCTS OF CYSTEINE DESULPHYDRASE OF *Pseudomonas salinaria*

H_2S, μM.	Pyruvic acid, μM.	NH_3, μM.	Ratio, H_2S: pyruvate: NH_3
0.59	0.64	1.41	1.00:1.08:2.39
0.28	0.22	0.19	1.00:0.79:0.68
0.47	0.51	0.96	1.00:1.09:2.0

NOTE: Three separate experiments; reaction period 3 hours in first two, 2½ hours in third.

Experimental conditions as described under Methods; 100 μg. pyridoxal phosphate and 80 μM. cysteine per vessel, KCl 3.2 *M*. All values have been corrected for production in the absence of cysteine.

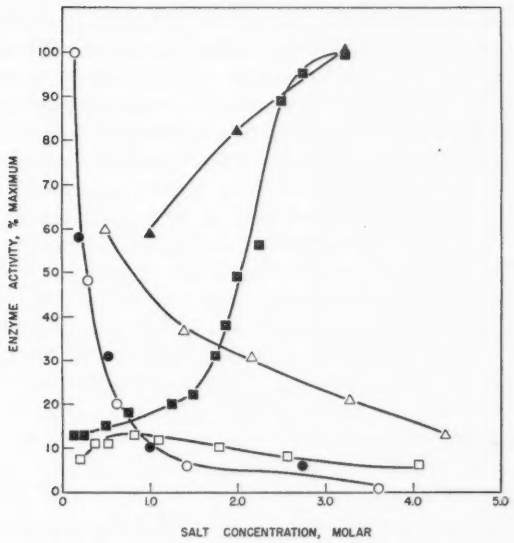

FIG. 1. Effect of sodium and potassium chloride on the cysteine desulphydrases of *Pseudomonas salinaria* and *Escherichia coli*. Open symbols denote sodium chloride, closed symbols potassium chloride.

●, ○: *E. coli*, 2 hour reaction period; 10 μg. pyridoxal phosphate and 0.2 mM. sodium phosphate buffer per vessel.

▲, △: *P. salinaria*, 2½ hour reaction period; 100 μg. pyridoxal phosphate and 0.2 mM. sodium phosphate buffer per vessel.

■, □: *P. salinaria*, 4¾ hour reaction period; 100 μg. pyridoxal phosphate and 0.1 mM. sodium phosphate buffer per vessel.

The sodium chloride concentrations shown in the figure include the sodium ion contributed by the buffer and (in the experiments with *P. salinaria*) by the salt in the extract. This amounted to about 0.1 *M* in the experiment with *E. coli*, and to 0.45 *M* and 0.2 *M* respectively in the 2½ and 4¾ hour experiments with *P. salinaria*. Since the cells contain potassium, a small concentration of potassium ion was also contributed by the extract. This has not been taken into account, but was probably less than 0.1 *M* in the *P. salinaria* experiments, and much less in the *E. coli* experiment.

The cysteine desulphydrase of *P. salinaria*, like that of *E. coli* (8), requires pyridoxal phosphate. In the presence of 0.78 *M* sodium chloride and 2.75 *M* potassium chloride only 0.025 μM. hydrogen sulphide was produced in 2 hours in the absence of pyridoxal phosphate. However, in the presence of 2, 10, 20, 50, and 100 μg. pyridoxal phosphate*, 0.08, 0.10, 0.12, 0.14, and 0.18 μM. hydrogen sulphide were produced.

The cysteine desulphydrase of *P. salinaria*, like other enzymes of this organism (2, 3), is most active in the presence of high concentrations of potassium chloride (Fig. 1). The enzyme is not completely inactive in the absence of potassium chloride because the buffer and the salt in the extract contributed a small quantity of sodium ion which caused a slight activation. The decline in activity with decreasing salt concentration is dependent on the length of the reaction period, being more rapid with a reaction period of

*Obtained from the California Foundation for Biochemical Research.

$4\frac{3}{4}$ hours than with a reaction period of $2\frac{1}{2}$ hours. Although the $2\frac{1}{2}$ hour experiment and the $4\frac{3}{4}$ hour experiment were performed with different extracts, they are probably comparable because we have always found the behavior of enzymes of *P. salinaria* to be reproducible under the same conditions with different preparations. To facilitate comparison, the quantity of hydrogen sulphide produced in the highest concentration of potassium chloride has been designated arbitrarily as 100% in each experiment. The response of the enzyme to sodium chloride was even more dependent on the length of the reaction period. With a $2\frac{1}{2}$ hour reaction period the maximum quantity of hydrogen sulphide was produced in 0.45 *M* sodium chloride (the lowest concentration that could be tested), and was about 60% of that obtained with the highest concentration of potassium chloride. With a reaction period of $4\frac{3}{4}$ hours the maximum production of hydrogen sulphide in sodium chloride occurred at a concentration between 0.5 and 1.0 *M*, but the amount produced was only about 12% of that obtained with the highest concentration of potassium chloride. In contrast to the enzyme of *P. salinaria*, the cysteine desulphydrase of *E. coli* was most active in the absence of salt and was markedly inhibited by relatively low concentrations (Fig. 1).

To determine whether *P. salinaria* fails to produce cysteine desulphydrase during growth at low salt concentrations, cells were harvested after 6 days' incubation from media containing 15, 20, 25, and 30% sodium chloride. They were resuspended to roughly equal cell concentrations and tested for cysteine desulphydrase activity in solutions of the same salt concentrations as the growth media. Cysteine desulphydrase activity was found in all. The amounts of hydrogen sulphide produced (0.54, 0.46, 1.01, and 0.34 μM. in 15, 20, 25, and 30% salt respectively) are not strictly comparable, since the amounts of enzyme were not the same, and the activities were measured at different salt concentrations. However, they do indicate that the enzyme is present in cells grown at each concentration and that the enzyme is active in whole cells at each concentration.

Discussion

Pseudomonas salinaria produces hydrogen sulphide by the action of a cysteine desulphydrase which appears to differ from the corresponding enzyme of *E. coli* only in its requirement for potassium chloride. In its preferential activation by potassium this enzyme resembles other enzymes of *P. salinaria* (2, 3). The cysteine desulphydrase of *P. salinaria*, like other enzymes of this organism, undergoes an irreversible inactivation at low salt concentrations. It is probable that sodium is less effective than potassium in preventing this inactivation, so the difference in the extent of the reaction in equimolar concentrations of the two salts increases with increasing time. Similarly with the longer reaction period the more rapid decline in hydrogen sulphide production with decreasing potassium chloride concentrations reflects the more rapid inactivation of the enzyme at the lower concentrations.

Cells of *P. salinaria* grown in four salt concentrations and tested at the same concentration as that in which they had been grown showed cysteine desulphydrase activity of the same order of magnitude. Therefore, the failure of growing cultures of *P. salinaria* to produce hydrogen sulphide at salt concentrations of 20% or lower cannot be attributed to absence of the enzyme or to failure of the enzyme to act in cells exposed to lower salt concentrations. Since the proteolytic activity of the organism is reduced at low salt concentrations (7), it may be that cysteine is not liberated from peptide linkages, or is liberated at such a rate that it is all incorporated into cellular protein.

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FORMATION OF EXTRACELLULAR LYSINE BY USTILAGO MAYDIS AND GLIOCLADIUM SP.[1]

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Abstract

A strain of *Gliocladium* sp. and a strain of *Ustilago maydis* produced approximately 400 μg./ml. of extracellular lysine in shaken Erlenmeyer flasks. In *U. maydis* growth media, most of the extracellular lysine, arginine, and histidine is in the free form. Metabolic studies show the lysine to be formed during early growth, concurrent with the formation of most of the extracellular organic nitrogen. The extracellular organic nitrogen and lysine is used during a late growth surge in experiments showing high lysine concentrations but not in those showing low lysine concentrations.

None of the compounds tested as precursors or intermediates resulted in an increased concentration of extracellular lysine.

Introduction

Microbial cells or the extracellular organic nitrogenous compounds formed by them are possible sources of amino acids.

The formation of extracellular organic nitrogenous substances by a number of fungi has been studied by Morton and Broadbent (4), whose paper should be consulted for prior references. These workers found most of the organic nitrogen in the extracellular metabolism solution to be in peptides with not more than 10% of the nitrogen in the α-amino groups of free amino acids. The possibility of obtaining specific amino acids from fungi was investigated by Richards and Haskins (7). Their screening studies revealed a number of fungi that concentrated lysine free in the medium during submerged aerated growth.

The present study was directed primarily toward the formation of extracellular lysine. The amount of lysine in the cellular peptide or proteins has not been determined. There is, however, little free lysine within the cell.

Materials and Methods

The nine cultures originally tested for lysine production were recommended by Dr. M. Richards (7) and included two strains of *Ustilago maydis*, two cultures of *Gliocladium* (probably *G. roseum*), one strain each of *Monochaetia unicornis*, *Darluca filum*, *Trichoderma viride*, *Streptomyces* sp., and an unidentified fungus. When tested in two media that differed in having $(NH_4)_2HPO_4$ and urea as sole nitrogen sources, two of the cultures, i.e. *Ustilago maydis* PRL 1092 and *Gliocladium* sp. PRL 233, were markedly superior and were selected for further study. Richards previously had found strains of *Ustilago* species to be the most promising extracellular lysine producers (7).

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The vegetative inoculum for all experiments was developed in 250 ml. Erlenmeyer flasks containing 50 ml. of the following medium: *d*(+)-dextrose (sterilized separately by autoclaving), 50 g.; $(NH_4)_2HPO_4$, 5 g.; K_2HPO_4, 1 g.; $MgSO_4.7H_2O$, 0.1 g.; KCl, 0.5 g.; $FeSO_4.7H_2O$, 0.02 g.; $ZnSO_4.7H_2O$, 0.01 g.; and distilled water to 1 liter. The experimental medium contained the following inorganic salts per liter of distilled water: K_2HPO_4, 0.3 g.; KH_2PO_4, 0.2 g.; $MgSO_4.7H_2O$, 0.2 g.; NaCl, 0.1 g.; $CaCl_2.2H_2O$, 0.1 g.; $FeSO_4.7H_2O$, 0.015 g.; and $ZnSO_4.7H_2O$, 0.01 g. The nitrogen source was varied qualitatively and quantitatively and was sterilized separately from the other medium ingredients. Varying concentrations of dextrose served as the carbon source. The organisms were grown in 250 ml. Erlenmeyer flasks and, like the inoculum, incubated at 27°–28° C. on a rotary shaker moving at 225 r.p.m. and describing a circle 2 in. in diameter.

For study of metabolic changes, flasks were harvested at intervals. The pH of the whole broth was taken immediately. Growth was estimated by pipetting 5 or 10 ml. of the whole broth into tared centrifuge tubes, washing the cells at least twice with distilled water, drying them to a constant weight at 90° C., and weighing the dried cells. The supernatant following centrifugation was subjected to the following analysis. The residual dextrose was determined by the colorimetric procedure described by Neish (5). Total nitrogen was measured by the micro-Kjeldahl method of Johnson (3) and ammonia nitrogen by Conway and Byrne's microdiffusion method (1). The organic nitrogen was calculated as the difference between the total nitrogen and ammonia nitrogen. Extracellular free lysine was assayed using *Leuconostoc mesenteroides* P60. Lysine in the intracellular free amino acid pool was also assayed after the cells were boiled for 20 minutes (8).

Other experimental details will be given in the appropriate sections.

Results

Developmental Studies

Gliocladium sp. PRL 233

Exploratory experiments had shown autoclaved urea to be an excellent source of nitrogen for lysine production and this was confirmed in the following experiment. Autoclaved and Seitz-filtered urea, $NaNO_3$, $(NH_4)_2HPO_4$, and NH_4NO_3 were used as single nitrogen sources. The results in Table I show urea, particularly autoclaved urea, to be the superior nitrogen source for lysine production. The superiority of autoclaved urea may be due to the release of ammonia during sterilization resulting in a favorable mixture of ammonia nitrogen and urea nitrogen.

With autoclaved urea as the sole nitrogen source and dextrose as the carbohydrate, the effect of varying the carbon and nitrogen levels was studied. The results are in Table II. Increased levels of extracellular lysine were obtained with increased levels of both dextrose and nitrogen, the highest lysine concentration occurring at the highest dextrose and urea concentrations used. Increasing the levels of dextrose and urea above the highest employed in this experiment has not resulted in more extracellular lysine.

TABLE I

NITROGEN SOURCES FOR LYSINE PRODUCTION BY *Gliocladium* SP.

Nitrogen source	μg./ml. of lysine after:		
	4 days	7 days	10 days
Urea-filtered	33	58	175
Urea-autoclaved	28	115	232
$NaNO_3$	52	21	60
$(NH_4)_2HPO_4$	36	15	13
NH_4NO_3	21	20	10

NOTE: Five milliliters of 4-day-old growth were used to start each 250 ml. Erlenmeyer flask containing 50 ml. of medium. The medium contained a single nitrogen source equivalent to 2.8 mg. N/ml., dextrose at a concentration of 50 mg./ml., and the inorganic salts detailed under the section on Materials and Methods.

TABLE II

EFFECT OF CARBON AND NITROGEN CONCENTRATIONS ON LYSINE FORMATION BY *Gliocladium* SP.

Dextrose, %	Urea, mg. N/ml.	μg./ml. of lysine after:		
		4 days	7 days	9 days
2.5	1	4	10	7
2.5	2	64	110	151
2.5	3	39	106	120
5	1	20	9	16
5	2	31	76	127
5	3	53	140	170
7.5	1	22	13	8
7.5	2	55	70	80
7.5	3	64	193	147
10	1	15	6	9
10	2	74	39	46
10	3	77	296	415

NOTE: Each 250 ml. Erlenmeyer flask contained 50 ml. of medium and was inoculated with 5 ml. of 5-day-old vegetative growth.

The results in Tables I and II indicate that the maximum lysine concentration may not have been reached, in some instances, at the time the experiments were terminated. Later experiments have shown the maximum extracellular lysine concentration, 400 μg./ml., to occur in 10 or 11 days. Hence the values in Tables I and II are at or very near the maxima.

Simultaneous experiments with *U. maydis* PRL 1092 showed that it produced somewhat more lysine and produced it earlier than the *Gliocladium*. Hence work with the *Gliocladium* was suspended in order to concentrate on the *Ustilago* culture.

Ustilago maydis PRL 1092

A number of single nitrogen sources at a level of 2.8 mg. N/ml. were tested for ability to support extracellular lysine production by this organism. The results are in Table III. The superiority of $(NH_4)_2HPO_4$ as a nitrogen source is obvious.

Using dextrose as the carbon source and $(NH_4)_2HPO_4$ as the nitrogen source, the effect of varying levels of carbon and nitrogen was studied. The results are in Table IV.

As with the *Gliocladium* culture, the highest extracellular lysine was obtained in treatments with the highest concentrations of carbon and nitrogen. Media with carbon and nitrogen concentrations higher than those used in this experiment support no higher concentrations of extracellular lysine. Such media may result in larger concentrations of total extracellular organic

TABLE III

NITROGEN SOURCES FOR LYSINE FORMATION BY *Ustilago maydis*

Nitrogen source	μg./ml. of lysine after:			
	1 day	2 days	3 days	5 days
Urea, Seitz-filtered	58	32	28	35
Urea, autoclaved	23	66	132	149
$NaNO_3$	20	40	29	7
$(NH_4)_2HPO_4$	18	251	155	5
NH_4NO_3	20	32	34	11

NOTE: Each 250 ml. Erlenmeyer flask contained 50 ml. of medium and was inoculated with 5 ml. of 2-day-old cells. The medium contained a single nitrogen source equivalent to 2.8 mg. N/ml., dextrose at a concentration of 50 mg./ml., and the inorganic salts detailed under the section on Materials and Methods.

TABLE IV

EFFECT OF CARBON AND NITROGEN CONCENTRATIONS ON LYSINE FORMATION BY *Ustilago maydis*

Dextrose, %	$(NH_4)_2HPO_4$, mg. N/ml.	μg./ml. of lysine after:			
		1 day	2 days	3 days	4 days
2.5	1	37	18	161	133
2.5	2	8	19	25	10
2.5	3	8	11	16	7
5	1	40	27	32	25
5	2	7	16	189	23
5	3	10	12	177	235
7.5	1	38	26	19	15
7.5	2	6	25	205	54
7.5	3	4	12	315	185
10	1	43	23	19	10
10	2	5	18	200	61
10	3	5	15	393	395

NOTE: Each 250 ml. Erlenmeyer flask contained 50 ml. of medium and was inoculated with 5 ml. of 2-day-old cells.

nitrogen, however. In all of the following experiments, a medium containing 10% dextrose and 1.416% $(NH_4)_2HPO_4$ (3 mg. N/ml.) was used in addition to the inorganic salts listed under Materials and Methods.

The volume of medium could markedly affect the aeration and agitation imparted to the culture. The effect of aeration and agitation was studied by using volumes of medium increasing from 30 ml. to 80 ml. per 250 ml. Erlenmeyer flask. The results are detailed in Table V. The 50 ml. volume of medium originally selected arbitrarily appears to be optimum for production of extracellular lysine and has been used in the following experiments.

TABLE V

EFFECT OF MEDIUM VOLUME ON LYSINE FORMATION BY *Ustilago maydis*

Volume of medium, ml.	μg./ml. of lysine after:			
	1 day	2 days	3 days	4 days
30	313	488	526	430
40	211	448	608	402
50	163	222	636	325
60	127	203	534	281
70	86	168	444	266
80	89		317	232

NOTE: Each flask was inoculated with 10% by volume of cells developed for 64 hours.

The amount of inoculum used in the above experiments was 10% by volume of the medium inoculated. The effect of inoculum size was investigated by varying the inoculum between 0.5 ml. and 10 ml. per 50 ml. of medium. The results are in Table VI. The size of inoculum had an effect on the amount of extracellular lysine formed as well as on the time at which the maximum was reached. Of the inoculum sizes used, 5 ml./50 ml. or 10% by volume, as used in the foregoing experiments, was the best.

TABLE VI

EFFECT OF INOCULUM SIZE ON LYSINE FORMATION BY *Ustilago maydis*

Ml. of inoculum	μg./ml. of lysine after:						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
0.5				16	142	152	287
1			24	153	178	215	
2		28	31	259	192	212	
3	24	97	238	226	205		
4	23	30	233	353			
5	21	48	412	370			
6	70	371	348	291			
7	105	339	285	247			
8	108	238	292	234			
10	182	227	203	197			

The age of the inoculum in the above experiments varied between 48 and 64 hours. The following experiment illustrates the effect of age of inoculum on formation of extracellular lysine. Inoculum was developed for periods between 1 and 10 days and a volume of 10% used to inoculate the fermentation flasks. The results are in Table VII. There is no marked effect of age of inoculum on the maximum concentration of extracellular lysine. The 2- to 3-day-old inoculum used in the above experiments is as good as any. The age of inoculum had an effect on visible growth however. The 6-, 8-, and 10-day-old inoculum resulted in lighter growth after 1 day incubation than did the other inocula though this is not reflected in the lysine concentration. The 10-day-old inoculum required an additional day to produce good growth and this is reflected in the lysine concentration.

TABLE VII

EFFECT OF AGE OF INOCULUM ON LYSINE FORMATION BY *Ustilago maydis*

Age of inoculum, days	μg./ml. of lysine after:			
	1 day	2 days	3 days	4 days
1	8	339	330	324
2	7	305	287	316
3	7	328	289	296
4	6	323	257	307
6	7	306	286	250
8	2	297	307	310
10	5	78	323	263

The above experiments were designed to find shake flask conditions that supported relatively high concentrations of extracellular free lysine and to develop a reproducible fermentation. The former objective has been realized but not the latter. If one compares the quantity of extracellular free lysine in comparable optimum treatments in the foregoing experiments, one finds values from around 300 μg./ml. to over 600 μg./ml. This variation from experiment to experiment has persisted and values of 700–800 μg./ml. of extracellular lysine have been obtained. By using the optimum conditions found in the above experiments, however, we do have a system that produces sufficient extracellular lysine for other projected studies.

Metabolic Studies

In order to gain an insight into the metabolism during growth and formation of extracellular lysine, a number of physiological changes were followed. A pattern that may be taken as typical in most aspects is shown graphically in Fig. 1.

Growth is accompanied by the utilization of dextrose and ammonia and a continual fall in pH. The extracellular organic nitrogenous compounds and lysine are formed during the early rapid growth. Between 48 and 60 hours there is a drop in dry cell weight. This decline has been found in different experiments resulting in high and low concentrations of extracellular lysine.

One can interpret this drop as due to autolysis although it is not clear why autolysis should occur at a time when residual dextrose and ammonia nitrogen are high. Just as perplexing is the growth surge that follows autolysis and occurs at a low pH.

The extracellular organic nitrogen increases at the time of apparent autolysis and decreases to approximately the previous level during the late growth surge. This late growth also uses the residual dextrose and some of the ammonia.

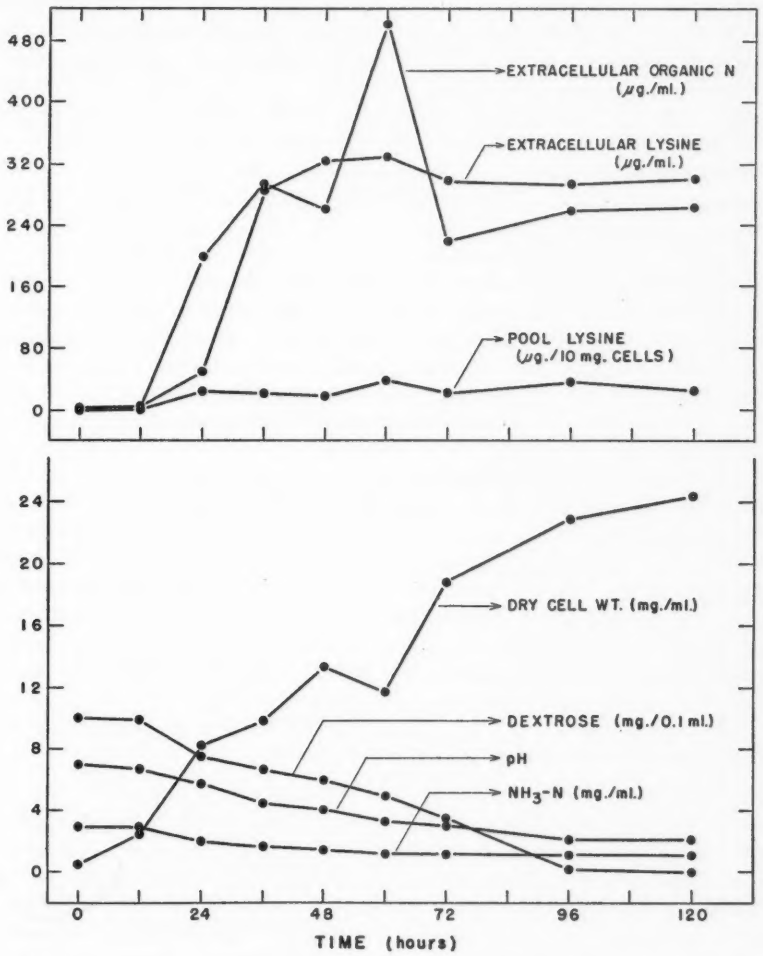

FIG. 1. Chemical changes occurring during growth and lysine formation by *Ustilago maydis*.

This increase and decrease in organic nitrogen points to the possibility that the organic nitrogen compounds released by autolysis are in a readily utilizable form and are the ones that disappear during later growth. The remainder of the organic nitrogen could be in non-available peptides. The extracellular lysine, however, does not change markedly during this time although there is a slight increase and decrease between 36 and 72 hours. Thus little free lysine could be released or used during autolysis and further growth.

The only apparent differences between experiments showing high and low concentrations of lysine are in the changes in extracellular organic nitrogen and lysine. Examination of an experiment resulting in 190 μg./ml. of extracellular free lysine showed a pattern quite similar in most aspects to that in Fig. 1. However, the extracellular lysine disappeared rapidly after 72 hours instead of remaining fairly constant. This was true likewise of the extracellular organic nitrogenous compounds. Thus, when the yield of lysine was low, both lysine and organic nitrogen were used during the late growth surge; and this did not occur when high concentrations of free extracellular lysine were obtained. This is merely an observable effect and more fundamental differences must be present.

One might interpret the above differences as due to the fact that, in the experiment with a high lysine concentration, the organic nitrogen occurs in non-utilizable peptides and, in the experiment with a low lysine concentration, it occurs mainly in free amino acids or simple readily available peptides. Some data contrary to this interpretation are available. The basic free amino acids in the 60 hour sample of the experiment showing a low lysine concentration were separated chromatographically and determined colorimetrically after treatment with ninhydrin. In addition to 190 μg./ml. of free lysine, there was 50 μg./ml. of free histidine and 473 μg./ml. of free arginine. These three amino acids contain 53% of the extracellular organic nitrogen. The amounts of other free amino acids were not measured but if only a low level of each were free the bulk of the extracellular organic nitrogen would be in free amino acids. A comparable study of the free basic amino acids in the fermentation depicted in Fig. 1 was not made. An analysis of another experiment yielding approximately 400 μg./ml. of extracellular free lysine was carried out, however (2). The organic nitrogen in this case was also contained largely in free amino acids.

The lysine of the free amino acid pool within the cells remains low and varies with no definite trend throughout the fermentation.

Precursors and Intermediates of Lysine Formation

The metabolic studies show active synthesis of extracellular lysine between 24 and 48 hours and cells of this age have been used for testing possible lysine precursors and intermediates. Both growing and resting cells were used. The tests with growing cells were made by addition of the compounds to flask cultures of the correct age. Resting cells were prepared by growing the culture under the optimum conditions for 48 hours, centrifuging the cells from each flask, and washing replicates with 50 ml. of buffer. The cells

TABLE VIII

EFFECT OF BUFFERS AND ADDED CARBON AND NITROGEN ON LYSINE FORMATION IN REPLACEMENT CULTURE

Treatment	μg./ml. of lysine after:			
	0 hr.*	8 hr.	24 hr.	41 hr.
Control	236	238	178	
Buffer pH 3.4		57	96	141
Buffer pH 3.4 + C and N		89	130	5
Buffer pH 4.4		142	0	0
Buffer pH 4.4 + C and N		86	13	0
Buffer pH 5.4		4	0	0
Buffer pH 5.4 + C and N		26	0	0
Buffer pH 6.4		2	0	0
Buffer pH 6.4 + C and N		5	0	0
Buffer pH 7.4		3	0	0
Buffer pH 7.4 + C and N		0	0	0

*0 hour represents time of washing and replacement of cells in buffer. Cells were 48 hours old at this time.

NOTE: Buffers are detailed in Reference 6.

from a flask were resuspended in 50 ml. of fresh buffer and the compound under test added to replicates. A series of buffers (6) was tested as well as the addition of 3 mg./ml. of dextrose and 2.32 mg./ml. of $(NH_4)_2HPO_4$ to replicates at each pH. The results are listed in Table VIII. Addition of extra carbon and nitrogen to the replicates had no advantage. The superiority of a pH 3.4 buffer, however, is marked and this buffer was used in all further tests.

The possible precursors that have been tested with both resting and growing cells include DL-α-aminoadipic acid, ε-amino-*n*-caproic acid, DL-ε-hydroxy-α-aminocaproic acid, and pipicolic acid, each at 2 mg./ml., and α,ε-diaminopimelic acid at 0.5 mg./ml. Other compounds tested were aspartic acid at 2 mg./ml. and citric, fumaric, malic, pyruvic, succinic, and acetic acids, each at 3 mg./ml. Aspartic, acetic, citric, fumaric, malic, succinic, and pipicolic were tested further by periodic additions to growing cells. The results need not be detailed for in no instance was there an increase in extracellular lysine. In addition, none of the compounds appeared to interfere with lysine formation.

Discussion

The strain of *U. maydis* used in these studies is capable of making at least 400 μg./ml. of extracellular lysine. The microbial assays as well as colorimetric assays following separation of the basic amino acids by chromatography show most if not all of this lysine to be in a free form. Likewise arginine and histidine are mostly in the free form. Further analysis of *U. maydis* growth media show that much of the total extracellular organic nitrogen is contained in free amino acids and probably even more is in small peptides that might invoke a partial response with the microbial assay organisms (2).

Why free amino acids should make up such a large proportion of the extracellular organic nitrogenous substances is unknown, as is the origin of the free amino acids. The lysine is obviously synthesized during early rapid growth of the culture. The other extracellular organic nitrogen is formed also at this time, and it has been surmised that this organic nitrogen is mainly in free amino acids. If these free amino acids were formed later by hydrolysis of extracellular proteins one would expect a different distribution of amino acids from that found (2).

The results show that microorganisms are a source of certain amino acids. Organisms that are far superior as producers of lysine and that produce different amino acids may be found by further screening. Thus the microbial production of amino acids for use as animal feed supplements as well as for other purposes might become economically practical.

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FACTORS AFFECTING SURVIVAL OF LYOPHILIZED FUNGAL SPORES AND CELLS[1]

R. H. HASKINS

Abstract

Spores of *Alternaria*, *Aspergillus*, *Fusarium*, *Gliocladium*, *Penicillium*, and *Pestalotia*, budding vegetative cells of *Ustilago*, and uredospores of *Puccinia graminis*, *P. helianthi*, and *Melampsora lini* were lyophilized using several techniques and various conditions. The best percentage survival of the lyophilized spores and cells was obtained when the initial freezing of the spore suspensions was just low enough to keep the suspension solid until evaporative freezing conditions were reached. Freezing to very low temperatures was unnecessary. Conditions permitting fast warming of the frozen suspension resulted in increased survival. A preliminary degassing treatment greatly enhanced percentage germination, as did the conditioning of the spores in a moist atmosphere immediately after the opening of the tubes for revival of the organisms.

In a previous study (2) it was shown that the percentage germination of *Aspergillus niger* spores following preservation by a centrifugal freeze-drying method was significantly higher than that following two more commonly used techniques each of which involved snap-freezing of the spore suspensions to low temperatures. These two methods have been termed the NRRL and the PRL methods (2). The former involves snap-freezing a suspension of spores in a suitable vehicle, evacuating the tube containing the suspension, first at the low freezing temperature, then eventually at room temperature, and finally sealing off the tubes containing the dried spore suspensions *in vacuo*. In the PRL method the spore suspensions are frozen in a freezer chest, followed by primary drying at room temperatures in a vacuum desiccator with secondary drying and sealing off *in vacuo* done on a suitable manifold arrangement.

If the better survival with the centrifuge method was due to the suspensions not being subjected to such low temperatures as with the other methods, then these other methods might be modified to give results equal to the centrifuge method, thus making the convenient but expensive centrifugal freeze-dry equipment less essential.

As a result of the investigation undertaken it was found that survivals of spores lyophilized by the so-called NRRL and PRL methods (2) could be increased to equal those resulting from the use of the centrifuge freeze-dry equipment by using less severe initial freezing temperatures and certain other modifications of technique.

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Issued as N.R.C. No. 4235.

Apparatus and Methods

The apparatus and methods used, except as noted, were essentially those described in a previous paper (2), with the bulk of the present work being done on a centrifugal freeze-drier.

The fungi used as test organisms were selected on the bases of spore type and availability. For small "dry" spores, the following genera were used: *Aspergillus niger*, PRL 326 (dark-colored, single-celled spores), *Penicillium*, PRL 151 (light-colored, single-celled spores), and *Alternaria*, PRL 472 (dark-colored, septate spores). For small "wet" spores, *Gliocladium*, PRL 1232 (light-colored, single-celled spores), *Fusarium*, PRL 1431 (light-colored, septate spores), and *Pestalotia*, PRL 1408 (dark-colored, septate spores) were used. *Ustilago maydis* (PRL 119) and *Ustilago* sp. (PRL 627) were selected as sources of vegetative cells. The uredospores of *Puccinia graminis*, *Melampsora lini*, and *Puccinia helianthi* were chosen as representatives of the large spores of phytopathogens.

The vegetative cells of *Ustilago* were pipetted from actively growing shake-flask cultures after 3 days' shaking at 225 r.p.m. at 28° C. Both freshly collected uredospores of the several rusts and collections stored at refrigerator temperatures were used in the work with phytopathogens.

For more efficient programing of the investigations, the germination times at various incubation temperatures were determined for each of the test organisms. With some of the organisms, freshly collected spores germinated more rapidly than did the lyophilized material.

Anastasiou (1) lyophilized spores harvested from *Aspergillus niger* cultures ranging in age from less than 3 to over 40 days and reported maximum survival with spores harvested at 5 to 6 days. However, in the present studies conidiospores were collected when production on a suitable sporulating medium had reached an apparent maximum.

No special attempt was made to have the various spore suspensions made up to a standard number of spores per milliliter of suspension. The volume of liquid used in each lyophile tube was approximately 0.2 ml.

The harvesting of spores and the preparation and filling of tubes were done in the usual manner, and procedures standardized as much as possible. Similarly, the techniques of opening of the tubes and revival of preserved organisms were standardized and controls included in each test. The percentage survival of spores was determined by direct count of germinated and ungerminated spores following standardized incubation conditions. At least 1000 spores were counted from each lyophile tube tested, with counts from three to eight tubes included in each average value.

Results

Temperature of Initial Freezing

The first step was to determine the temperatures reached by frozen suspensions drying in the centrifuge equipment.

With the centrifuge method, data on temperature changes in the spore suspension during centrifugation, evacuation, and drying are difficult to

determine. However, the temperatures reached by several different liquids during evacuation and drying in the primary drying chamber of a centrifugal freeze-drier were obtained.

Thermocouples were placed in the liquid in standard lyophile tubes and the liquid was then frozen, by partial immersion of the tubes in dry ice – alcohol baths, to a few degrees below zero (low enough that the liquid did not thaw before conditions for evaporative freezing were reached). The tubes were immediately subjected to vacuum in the primary drying chamber of the centrifugal freeze-drier. The thermocouples were connected through a vacuum-tight plug in the floor of the drying chamber to an eight-point recording potentiometer giving readings every 30 seconds.

While many varying types of runs were carried out, the type of data obtained is adequately illustrated in Fig. 1. During the period between the removal of the tubes from the freezing bath and the onset of evaporative freezing, the material in the tubes almost reached 0° C. With a four-tube load the frozen serum pellet reached about −12° C. for a short time while the distilled water pellet reached −25° C. but drying was completed in all tubes in less than 2 hours. A secondary drop in temperature occurred when the frozen pellet broke away from the sides of the tube, exposing more surface to drying. For a full 400-tube load, lower temperatures were reached for longer periods of time and complete drying took much longer, approximately 11 hours.

Serum, skim milk, and sucrose solutions did not reach as low a temperature as did distilled water but drying took longer.

Ideally, the temperature in the drying sample would approach that of the cold trap, but with the small samples used in lyophilization of cultures the surface exposed is limited and drying is completed before such temperatures

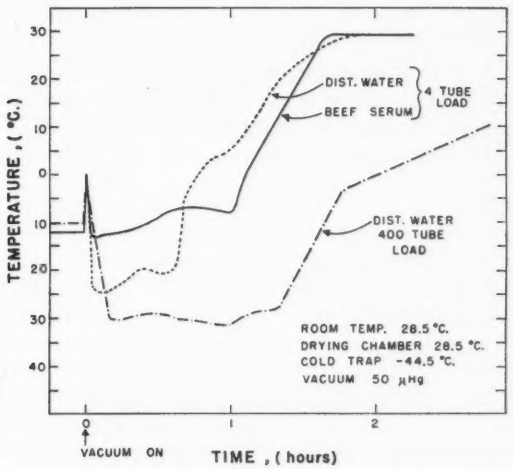

FIG. 1. Temperature changes in freeze-drying pellets in primary drying chamber of modified Edwards Model 3PS centrifugal freeze-drier.

can be reached. It is obvious, however, that temperature conditions with centrifuge freeze-dried samples are not as severe as those normally employed during snap-freezing in the NRRL and the PRL methods.

The second step was to examine the effect on spore survival of the temperature of initial freezing in the lyophile process.

Spores were lyophilized by the PRL method in beef serum, and in beef serum plus sucrose, using identical conditions except for the temperature to which the suspensions were initially frozen. The results (Table I) show that there was better survival when the less severe freezing temperature was used. The survivals of the spores lyophilized in serum plus sucrose were much better than those lyophilized in serum alone.

TABLE I

PERCENTAGE GERMINATION OF SPORES LYOPHILIZED IN BEEF SERUM AND IN SERUM PLUS SUCROSE BUT INITIALLY FROZEN TO DIFFERENT TEMPERATURES

	Percentage germination					
	Control (not lyophilized)		Frozen to −(16–18)° C.		Frozen to −(59–63)° C.	
Organism	In serum	In serum plus sucrose	In serum	In serum plus sucrose	In serum	In serum plus sucrose
Aspergillus	55.9	50.7	28.5	30.7	9.3	8.1
Fusarium	87.3	89.6	3.2	57.4	0	2.3
Gliocladium	99.1	99.4	0	32.3	0	27.4
Penicillium	99.4	94.4	1.9	60.7	1.8	40.6
Pestalotia	95.5	94.9	2.2	64.0	5.5	2.2

Duration of Initial Freezing

The length of time (from 5 minutes to 10 hours) that the suspension of *Aspergillus niger* spores was frozen at −50° C. before evacuating and drying has been shown not to affect significantly conidial survival (1). This was confirmed in the present studies.

Rate of Warming

Mazur (3) has shown the survival of spores of *Aspergillus flavus* suspended in distilled water and cooled rapidly to −70° to −75° C. to depend primarily on the rate of subsequent warming of the frozen suspension. Slow warming (3) was found to be more harmful than rapid warming when the spores were suspended in horse serum, aqueous NaCl, or aqueous sucrose, or in distilled water.

These results were confirmed in a comparison of the survival of the spores of six organisms frozen in water, in serum, and in sucrose solutions. The results with sucrose were similar to those obtained with serum (Table II). With spores frozen in water, the slower the warming from −65° C., the lower the percentage germination (Table II). With some organisms serum provided

TABLE II

PERCENTAGE GERMINATION OF SPORES SUSPENDED IN FROZEN SERUM AND IN FROZEN WATER FOLLOWING DIFFERENT RATES OF WARMING

(Percentages in frozen water in parentheses)

Organism	Rate of warming		
	Quick	Medium	Slow
Alternaria	38.4 (22.9)	48.8 (6.5)	3.9 (6.1)
Aspergillus	70.6 (54.0)	19.3 (31.6)	10.4 (18.3)
Fusarium	50.8 (67.7)	44.4 (43.9)	35.6 (30.7)
Gliocladium	90.1 (84.3)	87.8 (85.9)	72.8 (72.2)
Penicillium	78.8 (74.6)	83.0 (53.8)	56.9 (43.7)
Pestalotia	83.9 (60.5)	73.7 (53.2)	26.9 (31.7)

NOTE: Suspensions snap-frozen at −65° C. then warmed as follows:
Quick — 5 minutes; immersion in 35° C. bath.
Medium — 1 hour; left in air at 21° C.
Slow — 6 hours; left in freezing bath until it reached 21° C.

better protection than water but again slower warming resulted in poorer survival.

The survivals of vegetative cells of *Ustilago* sp. PRL 627 lyophilized in 10% sucrose by the NRRL method were not affected significantly by a range of freezing temperatures from −5° C. to −35° C. but were adversely affected by the rate of warming from the lower temperatures. In this experiment, the suspensions were held at the freezing temperature for the first 30 minutes of primary drying and then exposed to room temperature. The suspensions frozen at −31° C. were slowly warmed by being left in the freezing bath while it warmed to room temperature.

Duration of Secondary Drying

An investigation of the effects of varying the duration of secondary drying showed no great differences in percentage germination of each of the six test organisms over secondary drying duration ranging from 5 minutes to 24 hours. The secondary drying was over P_2O_5 and at pressures of 10 to 5 μ of mercury indicating that our primary drying was relatively complete and that the secondary drying system served mainly as a convenient means of sealing the lyophile tubes *in vacuo*.

Types of Carriers

Vegetative cells from shake-flask cultures of *Ustilago maydis* and *Ustilago* sp. were lyophilized in six different carriers with comparative results shown in Table III.

The vegetative smut cells were able to survive lyophilization in various carriers, but as with the spores of other fungi, best survival was obtained in carriers containing 10–20% sucrose (Table I).

While often the best survival was obtained when organisms were lyophilized in 10–20% sucrose, the use of such a suspending medium posed problems. When the less severe freezing temperatures were used, the suspension often

TABLE III

SURVIVAL OF VEGETATIVE *Ustilago* CELLS LYOPHILIZED IN DIFFERENT CARRIERS

Culture No.	Beef serum	20% sucrose	Klim	Skim milk	Sucrose + Klim	Sucrose + skim milk
PRL 119	+	+++	++	++	++++	++++
PRL 627	+	+++	++	++	++++	+++

Survival: light +; medium ++; heavy +++; very heavy ++++.

melted and boiled out of the tube or hardened into a thick sirup in which there was little or no survival. When the suspension suddenly dried as it started to foam, survival was excellent. Further, sterile water had to be added to the opened lyophile tube, on revival of the culture, to resuspend the spores for removal to nutrient media, a process not nearly as convenient as shaking a loose pellet from an opened tube on to or into a nutrient medium.

To make a more acceptable pellet, survival and pellet quality were examined using sucrose mixed with other materials. The addition of Klim or beef serum to the sucrose overcame some of the disadvantages of sucrose alone in pellet formation and still provided excellent survival by the centrifuge method (Table IV).

Most of the beneficial effects of the sucrose were retained when it was mixed with the more common suspending media such as beef serum or whole powdered milk (Klim). The advantages of the loose pellet and the increased survival in sucrose were both obtained. The commercially available whole powdered milk (Klim) was used because of the survival it afforded and its convenience over beef or horse serum.

The use of carriers is not always necessary and indeed may prove inadvisable as shown by Sharp and Smith (4). In the present work, wheat rust uredospores were found to survive both the PRL and centrifuge methods when no carrier was used, and also the centrifuge method when 20% sucrose was used as the carrier.

Prelyophilization Treatments

Anastasiou (1) has shown that the length of time and the temperature at which suspended conidia are kept, before freezing for drying, affects spore survival in the lyophile process. A long period and a high temperature of storage of the suspended spores prior to lyophilization result in rapid loss of viability after drying.

Subjecting the spore suspension to a vacuum immediately before freezing was shown (2, 5) to result in an increased survival of the lyophilized spores. This is true also for wheat rust uredospores when lyophilized with no suspending carrier. Subjecting the spores immediately before lyophilization to a low vacuum for 20 minutes resulted in a definite increase in survival (Table V).

TABLE IV

SURVIVAL OF VEGETATIVE *Ustilago* (PRL 627) CELLS LYOPHILIZED BY VARIOUS METHODS IN VARIOUS CARRIERS

Carrier	NRRL*	PRL	Centrifuge	Control
20% Sucrose	(Tr.)	(Tr.)	+++	+++
Sucrose + Klim	++	++	++++	+++
Sucrose + algin	0	(Tr.)	+++	+++
Sucrose + beef serum	+	++	++++	+++

Survival: Trace (Tr.); light +; medium ++; heavy +++; very heavy ++++.
*Frozen to −35° C. followed by slow warming during drying.

TABLE V

PERCENTAGE GERMINATION OF WHEAT RUST UREDOSPORES LYOPHILIZED BY MODIFIED CENTRIFUGE METHODS

Treatment before and after lyophilization	Approximate percentage germination	
	Two-year-old collection	Fresh collection
Control		
No treatment	0.1	100
No preliminary evacuation		
No conditioning in humid air	Trace	5
Conditioned in humid air	5	97–99
Preliminary evacuation		
No conditioning in humid air	0	15
Conditioned in humid air	10	98–99

The effect of the degassing procedure was studied by subjecting the spore suspension to various vacuums for various lengths of time. Pressures of 5, 10, 20, and 27 in. Hg, and times of 5, 10, 20, 60, and 120 minutes were used, with *Alternaria*, *Aspergillus*, *Fusarium*, *Gliocladium*, *Penicillium*, and *Pestalotia* as the test organisms. Results were inconclusive and the pre-evacuation treatment did not generally improve survival, though in several of the individual trials greatly increased survivals were obtained.

With sunflower rust uredospores as with many other fungi, percentage germination is increased if the spores are washed in distilled water (Table VI). Washing removes some substance that inhibits germination. The prelyophilization evacuation treatment, as well as the process of lyophilization itself, appears to do the same. In a number of rusts the lyophilized spores gave a greater percentage germination than did the unlyophilized control material.

Postlyophilization Treatments

The most obvious postlyophilization treatment is storage. Some cultures lyophilized by the author and his predecessors have been stored successfully

TABLE VI

PERCENTAGE GERMINATION OF LYOPHILIZED SUNFLOWER RUST UREDOSPORES FOLLOWING VARIOUS TREATMENTS

Control	
Unwashed	2– 5%
Washed	10–15%
Lyophilized	
Unwashed and "humidified"	30–40%
Washed and "humidified"	15–20%
Washed but not "humidified"	<1%

in a steel filing cabinet at ordinary room temperatures for 10 years. However, since these cultures were processed prior to the start of the present series of investigations, percentage survivals of spores immediately after lyophilization are not available. Such figures are now being obtained annually for other cultures stored at room temperature for comparison with a parallel series stored at refrigeration temperatures. Present indications are that survival is better in those preparations stored at the lower temperature than in those stored at room temperatures.

With rust uredospores conditions of revival are at least as important as conditions of lyophilization. If the dried spore suspension is "conditioned" or "humidified" by being placed in a humid atmosphere for about 20 minutes immediately on opening of the lyophile tube, improved germination results. The effect of this "conditioning" is well shown in Table V where germination is increased from 5–15% to 97–99%, and in Table VI. The optimum period for "conditioning" varies—with our sunflower rust uredospores about 6 hours resulted in maximum germination.

Discussion

With the greater survival afforded by the use of less severe temperatures in lyophile preservation of fungal spores and cells comes greater possibility of retention of all of the original characteristics of the organisms being lyophilized. It is becoming more apparent that with the use of suitable carriers where such are necessary, the application of preliminary evacuation and postlyophile conditioning techniques, and the use of less severe temperatures, the percentage survival of more fungi can be increased appreciably.

It appears that best survival is obtained when the initial freezing of the spore suspensions is only sufficiently low to keep the suspension solid until evaporative freezing conditions have been reached and that it is not only unnecessary, but also inadvisable, to freeze to very low temperatures. Conditions of drying should permit a fast warming of the frozen suspension. The length of time of secondary drying is not critical. For some fungi, at least, a preliminary degassing treatment greatly enhances percentage germination of lyophilized spores, as does conditioning of the spores in a moist atmosphere immediately after opening of the tubes for revival.

Acknowledgment

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AMINO ACIDS AND UTILIZATION OF SODIUM CASEINATE BY LACTIC STREPTOCOCCI[1]

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Abstract

The ability of strains of lactic streptococci to utilize unhydrolyzed sodium caseinate as the nitrogen source in an otherwise complete, chemically defined medium did not appear to be related to their minimal amino acid requirements. Sodium caseinate had a sparing effect on the requirement for some amino acids of some strains of streptococci that did not use caseinate, but the amino acids spared varied with the strain. However, leucine, isoleucine, methionine, and valine appeared to be essential for this group of organisms in the presence or absence of sodium caseinate.

Introduction

The ability of some lactic streptococci to utilize unhydrolyzed sodium caseinate as the nitrogen source in an otherwise complete, chemically defined medium was found to be a strain rather than a species characteristic (3). However, it was not known whether this difference in behavior among strains was due to differences in extracellular proteolytic activity or to differences in the amino acid requirements for growth. The present investigation was undertaken to determine if caseinate utilization was related to amino acid requirements.

Materials and Methods

Cultures of lactic streptococci* were maintained by weekly transfer in milk containing 0.5% glucose and 0.5% yeast extract (Difco). Cells used as inocula were from second subcultures in tryptone–tryptose broth (Medium 11(4)). After incubation for 18–24 hours at 30° C., the cells were centrifuged, washed three times, and diluted to their original volume in 0.9% saline. An inoculum of 0.1 ml. was used per tube of medium.

Amino acids were used in the concentrations recommended by Henderson and Snell (2). Various mixtures were dissolved in double strength basal medium (Table I) and the reaction adjusted to pH 6.5. Five milliliter aliquots were dispensed per tube and the volume made up to 8.5 ml. when sodium caseinate was to be tested; otherwise the volume was made to 9.5 ml. The tubes were autoclaved at 15 lb. pressure for 15 minutes. To the cooled medium was added 0.5 ml. of a Seitz filtered solution of asparagine and

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*_Streptococcus lactis_ 829 was from the Bacteriology Division, Science Service, Ottawa; strains 7962 and 7963 were from A.T.C.C.; and the various HP, HLC, and H22 cultures of _Streptococcus cremoris_ were isolated from commercial dairy starter cultures.

TABLE I

BASAL MEDIUM

	mg./5 ml.		μg./5 ml.
Lactose	200	Thiamine hydrochloride	4.0
Glucose	200	Pyridoxal hydrochloride	4.0
Sodium acetate	300	Pyridoxamine dihydrochloride	4.0
Sodium citrate	200	*p*-Aminobenzoic acid	4.0
K_2HPO_4	5.0	Riboflavin	8.0
KH_2PO_4	5.0	Nicotinic acid	8.0
$MgSO_4 . 7H_2O$	2.0	Calcium pantothenate	8.0
$MnSO_4 . H_2O$	0.4	Folic acid	0.2
$FeSO_4 . 7H_2O$	0.1	Biotin	0.04
Adenine sulphate	0.2	Vitamin B_{12}	0.0075
Guanine hydrochloride	0.2	Leucovorin (calcium salt)	2.5
Xanthine	0.2	Pantethine	2.5
Uracil	0.2	Thymidine	2.5
Orotic acid	0.25	pH 6.5	
Tween 80	10.0		

NOTE: All media contained 0.5 mg. asparagine and 0.5 mg. glutamine per 5 ml. of basal medium added as Seitz filtered solutions to the cold sterile medium.

glutamine (1.0 mg. of each per milliliter) and, when used, 1.0 ml. of a Seitz filtered solution of sodium caseinate (2.0 g./100 ml.). Sodium caseinate (Nutritional Biochemicals Corp.) was purified essentially as described previously (5).

After inoculation and incubation for 90 hours at 30° C., growth was measured by titrating the acid produced to the phenolphthalein end point. The results are reported as milliliters of 0.1 *N* NaOH per 10 ml. culture, corrected for the titration of the uninoculated control. A mixture of amino acids was considered "effective" when it produced 50% or more of the growth obtained with a complete amino acid mixture containing 18 amino acids (2). Strains of lactic streptococci have been designated as *caseinate utilizing* or *caseinate non-utilizing* depending upon their ability to grow in the basal medium (Table I) with sodium caseinate as the nitrogen source.

Results

All strains of lactic streptococci, whether capable of utilizing caseinate or not, required 6 to 12 amino acids for growth (Table II). In general, strains of *S. lactis* seemed to require fewer amino acids for growth than strains of *S. cremoris* but requirements varied from strain to strain. However, all strains tested required leucine, isoleucine, methionine, valine, arginine, and histidine. The ability of lactic streptococci to use sodium caseinate did not appear to be related to their amino acid requirements, since these strains as a group did not have simpler amino acid requirements than those not utilizing the protein. Thus, *S. lactis* 829 had the same minimal amino acid requirements as strain 7962 but only strain 829 grew when sodium caseinate was the source of nitrogen. The ability of these two strains to utilize peptides, however, appeared to be similar. Growth of both strains was the same

TABLE II

MINIMUM AMINO ACID COMBINATION FOR EFFECTIVE GROWTH OF LACTIC STREPTOCOCCI WITH AND WITHOUT SODIUM CASEINATE

Organism	Leucine	Isoleucine	Methionine	Valine	Arginine	Histidine	Phenylalanine	Proline	Glycine	Lysine	Alanine	Threonine	Tryptophan	Tyrosine	Cystine	Titer†	% maximum‡
Without sodium caseinate																	
S. lactis																	
829*	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	3.5	72.9
7962	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	3.3	76.7
7963	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	3.6	70.5
S. cremoris																	
H22-10*	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	3.4	82.9
H22-12*	+	+	+	+	+	+	+	+	+	+	0	+	0	0	0	2.6	63.4
HLC-1	+	+	+	+	+	+	+	+	0	+	+	0	0	0	0	3.7	74.0
HLC-6	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	2.6	86.6
HP-1	+	+	+	+	+	+	+	+	0	0	0	0	+	+	+	2.9	69.0
HP-3	+	+	+	+	+	+	+	+	0	0	0	0	+	+	+	2.6	65.0
With sodium caseinate (2.0 mg./ml.)																	
S. lactis																	
7962	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	3.5	—
7963	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	3.3	—
S. cremoris																	
HLC-1	+	+	+	+	+	+	+	+	0	+	+	0	0	0	0	4.1	—
HLC-6	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	3.0	—
HP-1	+	+	+	+	+	+	+	+	0	0	0	0	+	+	+	3.5	—
HP-3	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	2.0	—

NOTE: +, essential for growth; 0, not essential for growth in a minium amino acid mixture.
*Can utilize sodium caseinate without added amino acids.
†Milliliters of 0.1 *N* NaOH/10 ml. culture after 90 hours at 30° C.

$$\ddagger = \frac{\text{Titer with minimum amino acid mixture}}{\text{Titer with complete amino acid mixture}} \times 100.$$

whether the peptides, DL-leucyl-glycyl-glycine, DL-leucyl-glycine, L-leucyl-L tyrosine, DL-alanyl-DL-methionine, or glycyl-DL-valine, or the corresponding individual amino acids were supplied. Therefore, the differences in the ability of these strains to utilize caseinate did not appear to be related to their ability to utilize peptides.

All but two strains of streptococci that did not use caseinate grew with fewer amino acids in the presence of the protein than in its absence (Table II). The minimal amino acid requirements of the strains varied considerably and the amino acids that could be omitted when caseinate was present also varied. Leucine, isoleucine, methionine, and valine, however, appeared to be essential whether sodium caseinate was present or not.

Discussion

The essential amino acids for various strains tested agree closely with the findings of Niven (6) and Anderson and Elliker (1). Niven reported that the omission of leucine, isoleucine, methionine, valine, and arginine individually from a complete amino acid mixture exerted a pronounced effect on growth of strains of *S. lactis*, but the simplest combination that allowed prompt growth usually consisted of 14 amino acids. The strains of *S. lactis* used in the present study required fewer than 14 amino acids and in this respect, apparently, were less fastidious than those tested by Niven. However, their ability to grow with a limited number of amino acids may be related to the composition of the basal medium (Table I), which is nutritionally more complete than that used by Niven. Anderson and Elliker (1) in a similar nutritional investigation of 35 strains of *S. lactis* and *S. cremoris* showed that all strains required glutamine, leucine, isoleucine, methionine, valine, histidine, proline, and all but one required arginine.

The ability of lactic streptococci to utilize sodium caseinate was not related to their amino acid requirements. In fact, they did not necessarily require fewer amino acids than those that failed to utilize caseinate. Of two strains of *S. lactis* that had the same minimal amino acid requirements, only one utilized sodium caseinate. Also, neither of the strains of *S. cremoris* that used caseinate required fewer amino acids than those that did not use caseinate.

Some strains of streptococci that were unable to use sodium caseinate required fewer amino acids for growth in the presence of the protein than in its absence. This suggests that these strains obtained some but not all their essential amino acids from caseinate. The amino acids for which caseinate could substitute varied with the strain and this sparing effect apparently was not confined to any particular amino acid or group of amino acids.

Although all but two strains of lactic streptococci tested appeared to exhibit some proteolytic activity, differences in their ability to utilize sodium caseinate were apparent. These differences may involve the relative hydrolytic activity or the specificity of enzymes of various strains. Thus, the enzymes of non-utilizing strains may not yield a sufficient quantity of amino acids from caseinate to support growth, or these enzymes may fail to liberate some

essential amino acids. However, general conclusions are impossible because of the varied pattern of the amino acid requirements of various strains and of the sparing effect of sodium caseinate. A study of enzymes from these organisms should supply information to elucidate these points.

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OBSERVATIONS ON THE INITIAL CYTOLOGICAL EFFECTS OF BACTERIOPHAGE INFECTION[1]

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Abstract

Chromatin aggregation is the earliest cytological effect of several phage infections (e.g. P1, P2, and T7). This occurs at low multiplicities of infection if the medium contains a sufficient quantity of sodium chloride; on salt-deficient medium this aggregation no longer appears and is replaced by fragmentation. Infections with phages T2 and T5 do not normally result in the aggregation of the host chromatin, but this does occur when the multiplicity of infection is higher than three to six phages per cell and salt is present in the medium. The appearance of nuclear aggregation as the first cytological effect of infection under these circumstances adds an additional cytological stage. In the case of infection with T5 this is followed by chromatin disappearance, which is the normal first stage. Infections with T5, at high multiplicities, on salt-deficient agar cause chromatin fragmentation and no aggregation.

Chromatin aggregation was again the major alteration in cell structure when T2 bacteriophage "ghosts" were adsorbed. Since injection of phage DNA and phage multiplication do not occur under these conditions, it is concluded that adsorption alone is sufficient to cause this effect.

It is hypothesized that adsorption of phage alters the host cell surface so that the cell can no longer maintain an ionic equilibrium. The shift in internal ion balance is reflected in chromatin aggregation or fragmentation depending on the salt concentration of the medium.

Introduction

A recent paper from this laboratory (22) showed that the often observed aggregation of bacterial chromatin into compact masses and axial filaments is a function of the concentration of electrolytes in the environment of the cell. The effect could be prevented or reversed in an environment deficient in salts. It was hypothesized that chromatin aggregation is a consequence of the interaction of cations, particularly Na^+ and K^+, and polymerized desoxyribose nucleic acid, which behaves as an anionic gel. Thus, the phenomenon was considered as a disturbance of the normal electrolyte equilibrium within the cell.

Chromatin aggregation is a prominent feature of the earliest cytological effects of several bacteriophage infections. We were struck by the great similarity of the chromatin rearrangements observed in the experiments noted above and in the lysogenization of *Shigella dysenteriae* by phages P1 and P2 (20, 21). It seemed likely that phage might induce an electrolyte effect due to either a damage to the host cell surface or some characteristic of phage penetration and multiplication within the host cell. Some other bacteriophages, however, produce other initial effects, e.g. the centripetal movement

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Contribution from the Department of Bacteriology and Immunology, University of Western Ontario, London, Canada. The work was made possible by grants from the National Research Council of Canada. This work formed part of a thesis (20), which may be consulted for further details of experiment.

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of chromatin in T2 infections (11, 13) and the dissolution of chromatin in situ in T5 infections (14). Experiments showed that there was a common mechanism and that the cytological sequences observed in T2 and T5 infections are not immutable; the observations are recorded in this paper.

Materials and Methods

The *host cells* used were *S. dysenteriae* Sh, Sh/s, and their lysogenic derivatives (3, 4, 5, 6) and *Escherichia coli* B (8).

Bacteriophages P1 and P2 were isolated from the lysogenic strains of Sh and Sh/s in the manner outlined in a previous publication (21). Phages T2*r*, T5, and T7 (8) were strains maintained in this laboratory for several years. Adams (1) isolated several hybrids resulting from a mixed infection with T5 and PB phages. Two of these hybrids were obtained from the late Dr. Adams and are designated T5×PB, 1 and 2.

T2 bacteriophage-ghosts (9) were prepared and sent to us by Dr. A. F. Graham of the University of Toronto.

The *medium* used in this study was the LB medium of Bertani (3) and contained yeast extract, tryptone, glucose, and 1% sodium chloride. Salt-deficient LB agar was precisely the same as normal LB agar except that no sodium chloride was added.

Cells in the early logarithmic growth phase were infected on the surface of agar in the manner described in previous publications (14, 21).

The osmium–HCl–Giemsa procedure was used for the demonstration of the chromatin structures (13, 17, 21).

In order to study the effects of salt-deficient agar on chromatin aggregation, the cells were infected on normal LB agar; this provided a suitable environment for phage adsorption. As soon as the phage suspension had soaked into the agar, a large block of agar was removed and placed cell side down onto the surface of a well-dried salt-deficient LB agar plate. The block was quickly flicked from the surface of the deficient agar leaving an impression-transfer of the cells on the surface. Blocks were removed from this and the normal agar at intervals for fixation and cytological comparison.

Experimental

The normal chromatin arrangement is the same in *S. dysenteriae* Sh, Sh/s, and the lysogenic derivatives (21) and is represented in Fig. 1. The normal chromatin structures in cells of *E. coli* B in the early logarithmic growth phase have been described (13, 14).

Infection of *S. dysenteriae* Sh with phages P1 and P2 on normal LB medium produced the aggregation of chromatin that we had observed before (21). On salt-deficient LB agar, however, chromatin aggregation was replaced by fragmentation into a large number of small chromatin granules and, as time progressed, the cell became filled with chromatin. If the lytic course were to be followed, the granulation of the cell became more diffuse (Fig. 2). If

recovery of the cell took place, the granulation did not become diffuse, but, instead, reorganized into a complex reticulum of granules and threads (Figs. 3 and 4).

The last stages of infection prior to lysis resembled the corresponding stages in infections with T2 or T5 (13, 14) because the increase in chromatin showed as fine granules which were uniformly distributed throughout the cell. This was in marked contrast to the cytological stages on normal LB agar where the chromatin increase appears to occur along the elements of the chromatin reticulum in the expansion phase (21).

A similar experiment was performed using coliphage T7, a virus which causes aggregation in *E. coli* B at low multiplicities of infection (11). It was found that transfer of infected cells, with their chromatin condensed into sharply defined, small, rounded aggregates and short axial filaments, from normal LB agar to the deficient LB agar, resulted in the expansion and fragmentation of these chromatin aggregates. This disaggregation became cytologically evident during the first 15 minutes following transfer to the deficient agar. It is evident that the maintenance of aggregation in T7 infections requires a sufficient salt concentration in the medium.

Thus, the production of aggregation by phage infection requires the presence of salts in the medium, as does the aggregation resulting from various other circumstances (22). However, further experiments were needed in order to know whether aggregation was a consequence of phage multiplication or was a result of damage to the cell surface.

Although phage T2 causes margination of the host chromatin shortly after adsorption (11, 13), Bonifas and Kellenberger (7) observed the opposite effect, aggregation, following infection with the DNA-free T2 bacteriophage-ghosts, which consist essentially of the protein coats of the phage. If this were true, then aggregation could hardly be the result of injection of phage DNA into the host or multiplication of phage following its entry into the host.

E. coli B, *S. dysenteriae* Sh, and Sh($P2^{+++}$) were infected with T2 bacteriophage-ghosts (P:B > 10:1). When *E. coli* B, grown for $2\frac{1}{2}$ hours on normal LB agar at 37° C., had adsorbed the T2 ghosts the chromatin formed into spherical aggregates, which subsequently fused into axial filaments during the first 10 minutes after infection. By 30 minutes after infection, lysis of the host was well under way. Prior to lysis, the axial filaments fragmented and became less distinct. Cells of *S. dysenteriae* Sh underwent the same sequence of changes following infection with the ghosts.

Active T2 cannot multiply in Sh($P2^{+++}$), but the ghosts as well as the active phage can adsorb onto the surface of this lysogen (5). Infection of this strain with the ghosts caused the same sequence of changes observed in infected *E. coli* B and *S. dysenteriae* Sh and these changes are illustrated in Figs. 5 and 6.

It is apparent that phage multiplication is not a prerequisite for nuclear aggregation and that the act of adsorption alone is sufficient to cause aggregation. The importance of adsorption was clearly shown by the following series of experiments.

The Function of High P:B Ratios

While active T2 bacteriophage, at low multiplicities of infection, produced margination of the host chromatin structures followed by the development of granular DNA which eventually filled the cell (11, 13), infection with 70–100 phages per cell resulted in chromatin aggregation and premature lysis. Since the infected cells lysed around 5 minutes after infection, there was no time for the further fusion of the chromatin aggregates into axial filaments.

Cells of *S. dysenteriae* Sh carrying P2 prophage cannot support the multiplication of coliphage T5 or its hybrids T5×PB, 1 and 2 (19). Infection of Sh(P2) nevertheless results in the appearance of the characteristic first stage of infection, the "homogeneous cell" (14), in which there is cytological absence of chromatin. However, no further changes take place until lysis. When three or more of any of these phages were adsorbed by the host cell, nuclear aggregation preceded the disappearance of chromatin. The proportion of cells in the infected population showing chromatin aggregation before the usual fading of the chromatin (to form "homogeneous" cells) was a function of the phage to bacterium (P:B) ratio (Table I and Figs. 7 to 9), and steadily decreased with time. Thus, aggregation was a new phase introduced into the infection cycle. The proportion of homogeneous cells steadily increased because the normal infection cycle was blocked at this stage in Sh(P2), but in infections of Sh or Sh/s at high P:B ratios, the complete range of cytological changes (14) was observed to take place following the disappearance of the nuclear aggregates.

Since the salt sensitivity of nuclear aggregation in this situation resembled chromatin aggregation due to exposure to cold, U.V. irradiation, metabolic inhibitors, and starvation, it was felt that disturbances of the ionic equilibrium may play a major role. To study this, Sh was infected with T5 at a P:B ratio of 13:1, and the cells were transferred to salt-deficient LB agar.

TABLE I

THE EFFECT OF THE PHAGE:BACTERIUM RATIO ON THE CYTOLOGICAL EFFECTS OF INFECTIONS WITH T5 × PB HYBRIDS 1 AND 2 ON Sh(P2 *rd* l^+) ($P2^{++}c$)

Phage	P : B ratio	% aggregation	% homogeneous cells	Total No. cells counted
T5 × PB hybrid 1, 25 min. post infection	20 : 1	76.6	23.4	278
	2 : 1	14.9	85.1	467
T5 × PB hybrid 2, 30 min. post infection	10 : 1	28.4	71.6	461
	1 : 1	5.1	94.9	390*

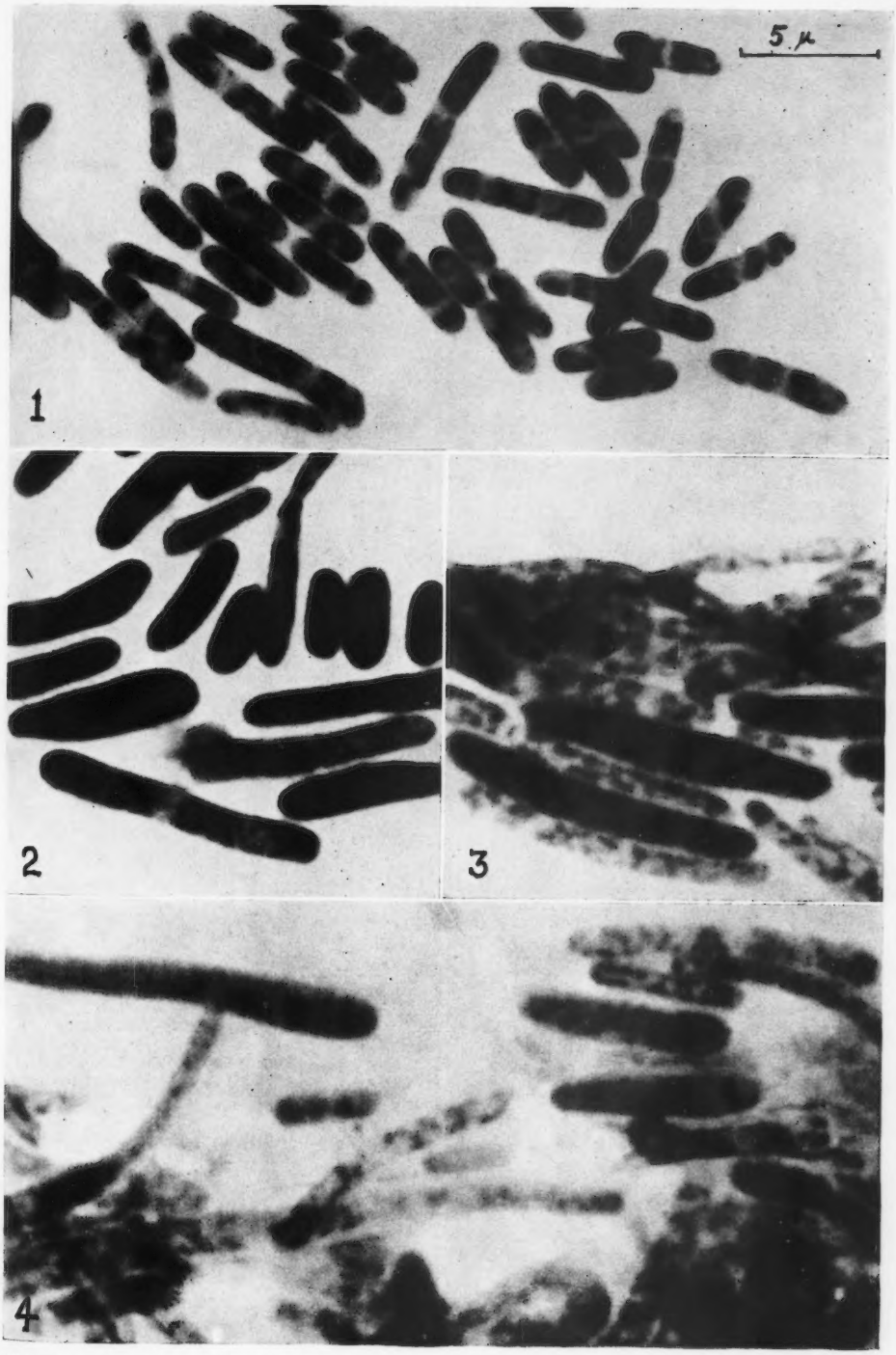
*Cells counted were those showing some abnormalities and presumed to be infected.

FIG. 1. Normal *S. dysenteriae* Sh/s, 3 hours at 37° C. on LB medium, hydrolyzed-Giemsa. The marker indicates magnification of all the figures.

FIG. 2. Sh/s, 60 minutes after infection with P1 temperate phage on salt-deficient LB medium, hydrolyzed-Giemsa.

FIG. 3. Same as Fig. 2 but 90 minutes after infection.

FIG. 4. Same as Figs. 2 and 3 but 150 minutes after infection.



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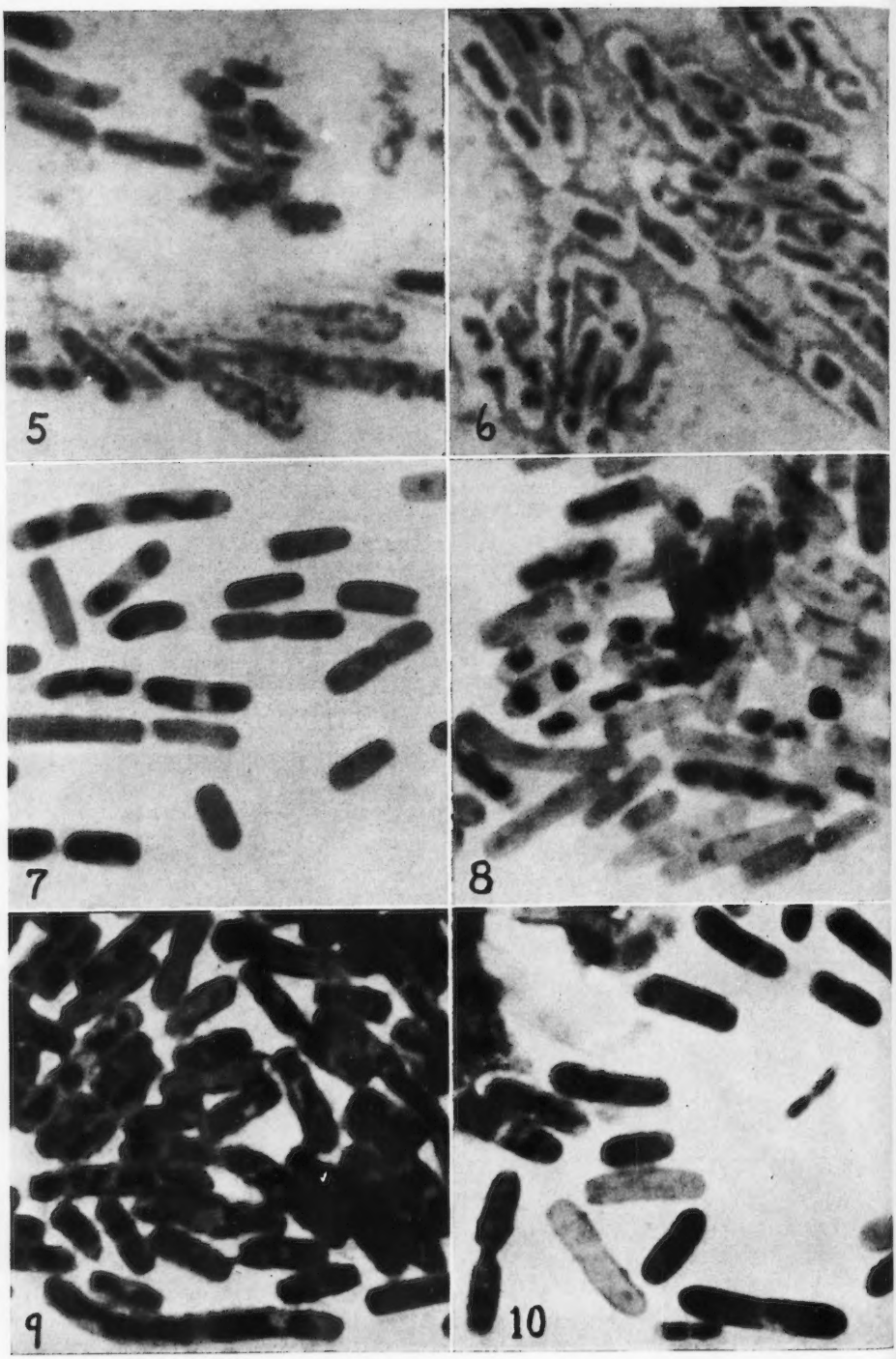

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TABLE II

THE CYTOLOGICAL EFFECTS OF INFECTION OF Sh/s WITH COLIPHAGE T5 AT A HIGH PHAGE TO BACTERIUM RATIO (13 : 1) ON NORMAL AND SALT-DEFICIENT AGAR 20 MINUTES AFTER INFECTION

Medium	Chromatin arrangement (% cells counted)			Total No. cells counted
	Homogeneous cells (no chromatin)	Aggregated	Partially aggregated or fragmented	
LB agar + 0.17 *M* NaCl	57.3	32.5	10.2*	267
LB agar, no added NaCl	55.5	0.5	44.0†	277

*All cells had partially aggregated nuclear structures.
†All cells had fragmented nuclear structures.

The results of such an experiment are recorded in Table II and Fig. 10. The proportion of the population showing cytological homogeneity on both the normal and deficient media was almost the same. On salt-deficient agar, the proportion of cells in the population showing nuclear aggregation was vanishingly small. There was fragmentation of chromatin instead of aggregation.

Discussion

In a previous publication (22) we have outlined the environmental conditions which appear to govern the conformation of the chromatin bodies of bacteria. If some form of damage is first done to the cell, rendering the cell incapable of maintaining an ionic equilibrium with the environment, and that environment contains cations such as Na^+ or K^+ in sufficient quantities, then the chromatin will aggregate; in a cation-deficient environment the chromatin will fragment.* Metabolic damage resulting from the action of dinitrophenol, antibiotics, U.V. irradiation, and total depression of metabolic activities by cold were shown to be effective. It is now apparent that similar ionic effects on chromatin occur during the early stages of phage infections.

The precipitating event in phage infections is unlikely to be phage multiplication since it occurs at a time in the cycle when no multiplication is taking place. Furthermore, the present studies and those of Bonifas and

*The term *fragmentation* is purely descriptive and should not be taken to mean an actual breakage of the elements of the chromatin body. What is observed as fragmentation may be only an extension of chromatin threads or expansion of a network with consequent separation of the stainable material, which may appear to be granular. This problem may be resolved by electron microscopy.

FIG. 5. Sh(P2), 10 minutes after adsorption of T2 ghosts on LB medium, hydrolyzed-Giemsa.
FIG. 6. Same as Fig. 5 but 50 minutes after adsorption.
FIG. 7. Sh(P2)(P2), 15 minutes after infection on LB medium with T5×PB1 at P:B ratio of 2:1, hydrolyzed-Giemsa.
FIG. 8. Same as Fig. 7 but 25 minutes after infection.
FIG. 9. Same as Figs. 7 and 8 but P:B ratio was 20:1 and 25 minutes after infection.
FIG. 10. Sh/s, 20 minutes after infection with T5 at P:B ratio of 13:1 on salt-deficient LB medium, hydrolyzed-Giemsa.

Kellenberger (7) clearly show that nuclear aggregation occurs as a result of adsorption of T2 bacteriophage-ghosts, which is a direct reversal of the state of affairs in normal T2 infections (10, 11, 13). The effect of T2 ghosts and of high multiplicities of infection with T2 and T5 phages leads one to suspect that the act of adsorption provides the initial stimulus since there is neither injection of phage desoxyribose nucleic acid nor multiplication of phage in the host cell in the case of the ghost infections. The cumulative effect of high multiplicities is also very suggestive of an effect on the cell boundary.

Puck and Lee (15, 16) showed that infection with any of the T phages initiates a cycle of permeability changes in the cell boundary during the period following attachment of the phage to the cell. This permeability change is cyclic. There appears to be a 'resealing' reaction, which requires the host's energy supplying systems, and its effectiveness would seem to depend on the degree of damage. Evidence of visible disorganization as well as chemical disorganization of cell membranes by phage adsorption has been provided by Weidel (18) and Barrington and Kozloff (2).

It is hypothesized, therefore, that adsorption of phage causes damage to the cell boundary to such an extent that ionic imbalance results. The imbalance is reflected by changes in chromatin arrangement, as we have postulated (22), due to either an increase in or a leaching of free cations around the nuclear structures according to the ionic environment of the cell. The destruction of the normal osmoregulatory capacities of the cell may include impairment of the 'metabolic pumps' which excrete Na^+ and conserve K^+ in the cell (12).

It has been considered that the sequence of cytological changes following bacteriophage infections is typical of the infecting phage and is immutable. The observations recorded in this paper show that this is not the case. The first cytological stage of infection may be modified by manipulation of the ionic environment (and the multiplicity of infection in some cases) in P1, P2, T2, and T7 infections. In the case of T5 a new initial cytological effect can be interposed between the time of infection and the normal first stage. The previously described initial effects of P1, P2, and T7 infections are certainly cation dependent but this is unlikely for T2 and T5 infections.

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EFFECT OF 4-AMINO-5-IMIDAZOLECARBOXAMIDE AND RELATED COMPOUNDS ON THE GROWTH OF ENTAMOEBA HISTOLYTICA[1]

MITSURU NAKAMURA

Abstract

4-Amino-5-imidazolecarboxamide and 4-amino-5-imidazolecarboxamidine were found to be stimulatory to *Entamoeba histolytica* when studied under bacteria-free conditions. These compounds in combination with folic acid or leucovorin, adenosine triphosphate, and ribose-5-phosphate greatly stimulated amoebic multiplication.

Introduction

The importance of purines in the growth of *Entamoeba histolytica* has been established (6, 8, 9, 10). However, little information is available regarding the biosynthesis of purines by the amoebas. Since folic acid or leucovorin is required in amoebic growth, it was thought that perhaps the amoebas are capable of closing the purine ring provided 4-amino-5-imidazolecarboxamide (AICA) and folic acid are furnished, thus eliminating the requirement of purines by the amoebas. The role of AICA in purine biosynthesis has been reviewed by Greenberg (5).

Materials and Methods

The effects of AICA, 4-amino-5-imidazolecarboxamidine (AICAR), and imidazole-4-carboxamide (ICA) were studied under bacteria-free conditions in a medium previously described by Nakamura (8). The NRS strain of *E. histolytica* used throughout these experiments was kindly supplied by Dr. Quentin M. Geiman, Stanford University School of Medicine, San Francisco, California. AICA was obtained from Dr. Joseph S. Gots; ICA from Dr. Karl Pfister; AICAR from Dr. M. Earl Balis; and folic acid and leucovorin from Dr. J. M. Ruegsegger.

Results

The data (Table I) show the amoeba-stimulatory activity of AICA and AICAR and the absence of such effects by ICA. When the basal media were fortified with serine or methionine in addition to AICA or AICAR, the growth of amoebas was still greater. Excellent amoebic growth occurred when AICA (or AICAR), serine (or methionine), and folic acid (or leucovorin) were combined. The addition of serine and methionine to amoeba cultures containing ICA did not increase amoebic multiplication. The slight stimulatory effects seen with folic acid and leucovorin in the presence of ICA may

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TABLE I

EFFECT OF 4-AMINO-5-IMIDAZOLECARBOXAMIDE AND RELATED COMPOUNDS ON THE GROWTH OF *Entamoeba histolytica*

Material assayed	Total no. of determinations	Growth of amoebas
Basal (control)	75	0
Basal + 4-amino-5-imidazolecarboxamide (AICA)	5	++
Basal + 4-amino-5-imidazolecarboxamidine (AICAR)	4	++
Basal + imidazole-4-carboxamide (ICA)	3	0
Basal + AICA + serine	4	+++
Basal + AICA + methionine	4	+++
Basal + AICA + methionine + folic acid	3	++++
Basal + AICA + methionine + leucovorin	5	++++
Basal + AICA + methionine + folic acid (or leucovorin) + adenosine triphosphate + ribose-5-phosphate	9	+++++
Basal + AICAR + serine	2	+++
Basal + AICAR + methionine	2	+++
Basal + AICAR + methionine + folic acid	3	++++
Basal + AICAR + methionine + leucovorin	3	++++
Basal + ICA + serine	4	0
Basal + ICA + methionine	4	0
Basal + ICA + methionine + folic acid	4	+
Basal + ICA + methionine + leucovorin	3	+

have been due to the growth factor activity of these compounds themselves since Nakamura and Baker (9) reported these compounds as growth factors for *E. histolytica*.

Discussion

These experiments suggest that the amoebas are capable of producing purines when supplied with purine ring precursors and a "1-carbon" carrier system. Much evidence has accumulated indicating that units of ribose and phosphate are added to the carboxamide prior to ring closure (1, 11, 2, 4). There is a possibility that the amoebas bring about ribosidation of purines and also of AICA since adenosine triphosphate and ribose-5-phosphate possess high stimulatory activity on this protozoan (8). Kornberg *et al.* (7) have described a reaction whereby adenosine triphosphate and ribose-5-phosphate yield 5-phosphoribosylpyrophosphate which in turn combine with adenine to form adenosine-5-phosphate. They have suggested that 5-phosphoribosylpyrophosphate may prove to be an intermediate in the synthesis of ribotides of acyclic purine precursors. It is of interest that maximum amoebic multiplication occurred when AICA, methionine, leucovorin, adenosine triphosphate, and ribose-5-phosphate were all combined.

Since preformed purines also possess a stimulatory effect on the amoebas (9), it is conceivable that in *E. histolytica* there is a coexistence of two mechanisms for the synthesis of nucleic acids: (*a*) one formed from acyclic purine precursors, and (*b*) one formed from intact purines. A similar mechanism has already been postulated by Furst and Brown (3).

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THE INFLUENCE OF OXYGEN AVAILABILITY ON THE DEGREE OF NITRATE REDUCTION BY PSEUDOMONAS DENITRIFICANS[1]

V. B. D. SKERMAN AND I. C. MACRAE[2]

Abstract

An attempt has been made to correlate nitrate reduction by *Pseudomonas denitrificans* with oxygen concentration in solution.

Evidence has been obtained which indicates that nitrate reduction occurs only when the oxygen concentration is below the critical level at which the oxygen utilizing enzymes are saturated. Whilst precise data on the quantitative relationship between nitrate reduction and oxygen reduction at oxygen concentrations between 0 and 0.3 p.p.m. are lacking, it has been clearly established that nitrate reduction occurring in so-called "aerated" solutions is due mainly to the activity of cells deprived of an oxygen supply and that there is a direct relationship between nitrate reduction and the oxygen solution rate. No reduction was detectable at an oxygen concentration above 0.2 p.p.m.

It has also been shown that with gas dispersed at the bottom of a fluid the breaking of the foam accumulating on the surface contributes markedly to the oxygen concentration in the solution.

Procedures employed in the study are described in detail and implications of results on nitrate reduction in soils have been discussed.

Introduction

In a previous publication (Skerman and MacRae (11)) the authors showed that when a suspension of *Pseudomonas denitrificans* possessing a well-developed nitratase system was aerated to the extent that a measurable concentration of oxygen was maintained in the solution throughout the experiment, no nitrate was reduced. When commercial nitrogen containing a small amount of oxygen was bubbled through the suspension there was a partial inhibition of nitrate reduction although it was not possible to detect any oxygen in the solution by the method of analysis employed. Since continual exposure of the cells to oxygen partial pressures approximately equal to that of the sterile solution in equilibrium with air did not affect the subsequent nitrate reduction rate under anaerobic conditions, it was evident that the apparent inhibitory effect of oxygen on nitrate reduction was probably one of direct competition as indicated by Stickland (13).

In view of the fact that complete inhibition occurred at an oxygen concentration equivalent to that in equilibrium with a partial oxygen pressure of 0.008 in the gas phase, it was necessary to devise some method whereby the competitive action could be studied at levels lower than this. One possibility was to prepare gas mixtures having a range of oxygen concentrations between 0 and 1% and to attempt to establish a near equilibrium with these gas mixtures. To establish such equilibria would have necessitated the use of gas flow rates which were quite impracticable. Passage of 100 ml. of air per

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minute through a No. 3 porosity sintered glass disk into 50 ml. of solution was considered to be the upper limit at which an experiment could be conducted without major loss of solution and, as Chain *et al.* (3) have shown, the oxygen diffusion rate with increased gas flow tends to reach a limit.

An alternative procedure was to attempt to correlate the final steady state oxygen levels (Wise (14), Hixson and Gaden (5), Bartholemew *et al.* (1), Finn (4)) with the reduction of nitrate. This could be done either by choosing a single gas mixture and varying the rate of flow or by maintaining the latter constant and varying the gas mixtures. Both of these methods were eventually employed.

The fact that the inhibition was occurring at oxygen concentrations considerably lower than that reported by Sacks and Barker (9) and Marshall *et al.* (7) also made it desirable that continuous measurement of oxygen be made in the solution itself. The external sampling technique described in the previous paper was not suited for extremely low oxygen concentrations. Despite the rapid sampling rate and the magnification of the oxygen diffusion current caused by turbulence, it was not possible to obtain a measure of the oxygen at very low levels.

Chain *et al.* (3), Longmuir (6), and Moss (8) have all employed a rotating gold or platinum electrode. According to Longmuir and Moss the electrode requires aging at a fixed potential before accurate readings are obtained. Chain *et al.* do not mention this characteristic. Some preliminary tests with a rotating electrode were made with only limited success. Since the experiments were to be conducted in a 30-minute interval, the necessity to age the electrode made it unsuitable.

Bartholemew *et al.* (1) made use of the dropping mercury electrode screened with stainless steel mesh and immersed in the solution. A modification of this was used by the authors in the present study.

Materials and Methods

As Chain *et al.* point out, the stainless screen as used by Bartholemew *et al.* is subject to blockage. In this study a cylindrical perspex screen (Fig. 1) which was supported on the stem of the saturated potassium chloride bridge within the cell was used instead. The base of the screen formed a small cup of *ca.* 0.5 ml. capacity which retains the mercury drops. The total capacity of the cup is only 1% of the total volume of solution used in any experiment. When assembled, the tip of the dropping electrode was set approximately halfway up the large hole in the side of the screen. In this position it was well exposed to the rapidly moving solution and in a 40 minute period the mercury drop remained well clear of the advancing surface of the pool accumulating in the base cup. The V-shaped base of the small ledge fixed to the base of the cylinder acted as a baffle and divided the stream of gas bubbles which reached it from the sintered glass plate completely preventing the gas from accumulating under it, and preventing bubbles from sealing the open end of the bridge. After leaving the plate, numerous bubbles tended to swing back

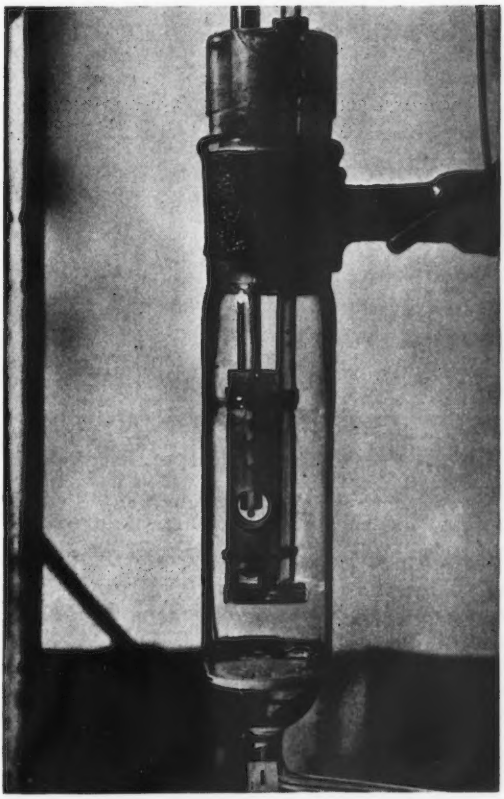


FIG. 1. The dropping electrode assembly and aeration cell used in the study.

towards the walls of the cylinder and entered through the large holes in the sides. If the electrode was set too high, e.g. in line with the upper edge of the hole, bubbles tended to settle on the broad electrode tip. The close proximity of the bubbles to the dropping mercury caused marked increase in the diffusion current. When set towards the middle of the hole, this defect was largely avoided although occasional bubbles still found their way to the electrode. Their presence was easily recognized from the characteristics of current changes.

The whole of the perspex shield was immersed below the surface of the liquid in the cell during operation. Visible observations showed that there was free transit of the liquid through the cylinder.

The outer end of the saturated potassium chloride bridge was immersed in the saturated potassium chloride of a saturated calomel electrode. The dropping electrode and anode were coupled to a Cambridge recording polarograph. Oxygen readings were taken at 0.6 volt v. S.C.E.

The Aeration Cell

Experiments were conducted in a cylindrical glass cell 3.5 cm. wide and 9.0 cm. deep fitted with a No. 3 porosity sintered glass disk at the base and a B29 ground glass joint at the top (Fig. 1). The dropping electrode and bridge were mounted in a perspex cone machined to fit the glass socket. Samples could be removed and reagents added through holes in the cone. When using such an assembly it is important to lightly grease the perspex cone to prevent it sticking.

Gas mixtures were introduced through the sintered disk after they had passed through a flowmeter and humidifier. The humidifier and aeration cell were immersed in a water bath at 28 ± 0.1° C. so that the menisci of contained liquids were well below that of the level of the water in the bath. The water bath itself was composed of perspex to permit continuous observation of the cell during operation.

Nitrate Estimation

The methods outlined in the previous paper (Skerman and MacRae (11)) were used throughout.

Bacterial Cells

Pseudomonas denitrificans was inoculated into a medium containing 1% Difco peptone, 0.5% Difco yeast extract, 0.5% NaCl, and 0.2% potassium nitrate. Cells were harvested after 24 hours at which time maximum nitratase activity is reached with the cultures. After removal from the refrigerated Sharples centrifuge the cells were washed twice with *M*/60 phosphate buffer at pH 7.0 and finally suspended in *M*/40 phosphate buffer at pH 7.0 to a density equivalent to *ca.* 5.0×10^9 cells per milliliter.

Substrate

Most of the work published in the previous paper was performed with Difco yeast extract as a substrate in a final concentration of 0.2% and a cell density of 2.5×10^9 per milliliter. With the increased density of cells employed it was found that the rate of oxygen uptake after 30 minutes' gassing with air was only about half the initial rate. An investigation of this led to the use of glutamic and aspartic acids in subsequent experiments. An outline of the investigations is given below.

In the final experiments glutamic acid was used at a final concentration of 1 mg. per ml. and aspartic acid at 1.5 mg. per ml. All substrates were neutralized before use.

Nitrate was used at a final concentration of 500 p.p.m. NO_3^-.

General Procedure

Prior to any experiment the gas mixture to be employed was allowed to flow through the flowmeter, humidifier, and cell at 100 ml. per minute for 10 minutes. Meanwhile the graph was placed on the photographic paper and the galvanometer zero recorded (Fig. 2A). Thirty-five milliliters *M*/40 phosphate buffer at pH 7.0, 5 ml. of substrate, and 5 ml. of 5000 p.p.m.

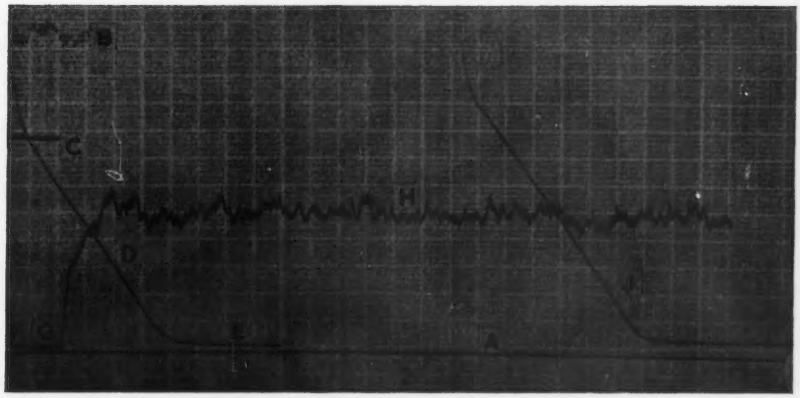


FIG. 2. A specimen polarogram illustrating measurements made during the experiments. For explanation see text.

NO_3^- were pipetted into the cell and the mixture flushed with the gas mixture at 100 ml. per minute for 10 minutes. This time was found to be sufficient to bring the oxygen in the solution into near equilibrium with that in the gas phase. A check was then made on the tip of the electrode to see that it was free of gas bubbles and the "agitated" oxygen level recorded (Fig. 2B). The gas flow was then cut off and the "stationary" oxygen concentration recorded (Fig. 2C). During this operation a slight pressure, insufficient to cause any bubbles of gas to pass through the sintered disk, was maintained to prevent backflow of the liquid.

Five milliliters of the cell suspension were then added and mixed by passing a slow stream of pure oxygen into the mixture through a pipette. Pure oxygen was necessary at this stage since air could not raise the oxygen level sufficiently high to register the uptake rate. The increment in oxygen concentration was observed on the galvanometer. When it approached that obtainable with air in the cell-free mixture the oxygen was cut off and the initial oxygen uptake rate of the population recorded (Fig. 2D). The recording was allowed to proceed a little beyond the point at which the residual current (*Ir*) level was reached (Fig. 2E). This indicated the "no oxygen" level at the beginning of the experiment. This was used for reference purposes in later parts of the experiment.

The recording camera was then returned to 0 and a portion of the residual current plotted (Fig. 2F). The recording speed was then changed to one-quarter of that used to register the oxygen uptake rate. (The Cambridge instrument allows the use of a 10 minute or 40 minute graph.) Antifoam was added to the cell. The recorder was then started, recording the residual current (Fig. 2G) until the gas mixture was fed into the cell at the desired rate. In some polarograms "F" was not recorded, the initial residual being taken from "G". A constant procedure was adopted in introducing the gas

mixture. Initially there was practically no pressure on the sintered glass plate. The pressure from the gas cylinder was increased rapidly till the flowmeter registered the desired flow rate. Because of the considerable back pressure from the sintered glass disk no gas flowed through the disk until this was overcome. This necessitated applying a pressure in excess of that required for final operation in order to maintain a constant flow rate to the humidifier which also acts as a pressure regulating tank. For the latter purpose a 250 ml. gas wash bottle was used. Once the gas flow through the plate began, the applied pressure had to be regulated until the flow rate remained constant. These preliminary operations were quite simple and occupied only 2–5 minutes. After this only occasional attention was necessary to maintain a constant flow. An identical procedure was followed in plotting oxygen absorption curves.

The first sample was removed for nitrate analysis approximately 2 minutes after the gassing rate reached its constant level. The oxygen concentration was continuously recorded during the subsequent 30 minute period (Fig. 2H). At the end of this period the second sampling was made for nitrate analysis.

The recorder was changed to the rapid speed and returned to a position suitable for recording a second uptake rate. The gas was cut off and the mixture reoxygenated as before with a slow steam of pure oxygen. The final uptake rate was then recorded (Fig. 2J), care being taken to see that the final residual current was plotted for comparison with the recording of oxygen concentration during the experiment. By this means it was possible to correlate oxygen concentration, substrate availability, and nitrate reduction.

In all polarograms except that of Fig. 33 the oxygen concentration in p.p.m. can be determined by subtracting the *Ir* value (in millimeters original scale) from the "stationary" or "agitated" oxygen limiting current reading and multiplying the former by 0.192 and the latter by 0.146. The time base in all polarograms is 20 seconds per bar at rapid speed and 80 seconds per bar at slow speed.

Results

Choice of Antifoam

In the previous paper the authors employed a sparger to break the foam formed during the gassing of the suspension in the cell after failing to control the foam satisfactorily with octadecanol in lard oil, the only non-toxic antifoam then available. Adoption of the technique outlined above necessitated a further search for a suitable antifoaming agent. The problem was finally overcome with General Electric Silicone Antifoam 60, a sample of which was kindly supplied by Professor W. H. Peterson of Madison, Wisconsin. Prior to the acquisition of this substance it was found that the foaming properties resided mainly in the cell suspensions. It was observed that if the scum formed after the addition of octadecanol was repeatedly removed a stage was reached where foaming ceased despite prolonged gassing of the remaining suspension. The population apparently consisted of two cell types which

were separated in the process. Determination of nitratase activity showed that the residual population possessed an activity per cell equal to the original.

Influence of the Antifoam on the Function of the Dropping Electrode

It was conceivable that the antifoam, although used in minute amounts, may affect the behavior of the dropping electrode. Such an effect has not been observed, the diffusion current in stationary solutions which have been aerated with antifoam present being the same as those aerated without it. The initial oxygen uptake curves in the illustrations were all performed on mixtures without antifoam and the final ones after the addition of antifoam. The identical nature of these curves where the substrate is not limiting indicates a lack of interference by the antifoam. The only trouble which was encountered occurred when an electrode was withdrawn and reimmersed after antifoam was used. This was due to surface scum adhering to the face of the electrode. With Silicone Antifoam 60, little trouble was experienced. In the general procedure outlined above, the electrode was never removed after the antifoam was added, until the experiment was completed.

Influence of Antifoam on the Oxygen Concentration in the Solution

In early experiments considerable difficulty was experienced in obtaining reproducible oxygen levels despite the fact that all manipulations appeared to be uniformly controlled. The variability was eventually traced to the antifoaming treatment. Figs. 3, 4, and 5 illustrate this effect quite dramatically. In the three experiments, air was passed through the solution at 25 ml. per minute. Glutamic acid was being used as the substrate. In Fig. 3 the cell and electrode assembly were very carefully washed with warm water to remove traces of the antifoam used in previous experiments. No antifoam was added to the mixture initially. The oxygen concentration rose quite rapidly to an apparent "steady state" concentration and remained at this level until Silicone Antifoam A was added at point A. The appearance of the cell prior to the addition of antifoam is shown in Fig. 6. The foam immediately disappeared on the addition of the antifoam at point A and the oxygen concentration was reduced sharply to the lower steady state level.

In Fig. 4 the cell had been given only a cursory rinse which failed to remove traces of antifoam from the glass surface and electrode assembly. The quantity of antifoam was not sufficient to completely retard formation of foam, which was restricted to a shallow layer of bubbles covering only a portion of the surface (Fig. 7). A much lower apparent "steady state" concentration was reached, which dropped sharply on the addition of more antifoam at point A.

In Fig. 5 sufficient antifoam was added to the mixture to suppress foam formation almost completely. Gas bubbles rising through the liquid broke through the surface with little tendency to form foam (Fig. 8). A very low steady state level was maintained throughout the experiment. (Note: The oxygen uptake curve in Fig. 5 was unfortunately not plotted beyond the point where it meets the steady state curve.)

Figs. 3 and 4 suggest that the breakdown of foam leads to a steady state level of oxygen concentration. This is purely accidental. Fig. 9 shows an experiment similar to that shown in Fig. 3 but using aspartic acid as the substrate. Although the respiration rate remained constant during the experiment the oxygen concentration prior to the addition of antifoam showed a progressive increase until antifoam was added. The apparent steady state level in Fig. 3 was probably due to the fact that this tendency to increase was counterbalanced by an increase in the rate of respiration which occurred when glutamic acid was used as a substrate (see Figs. 11–15).

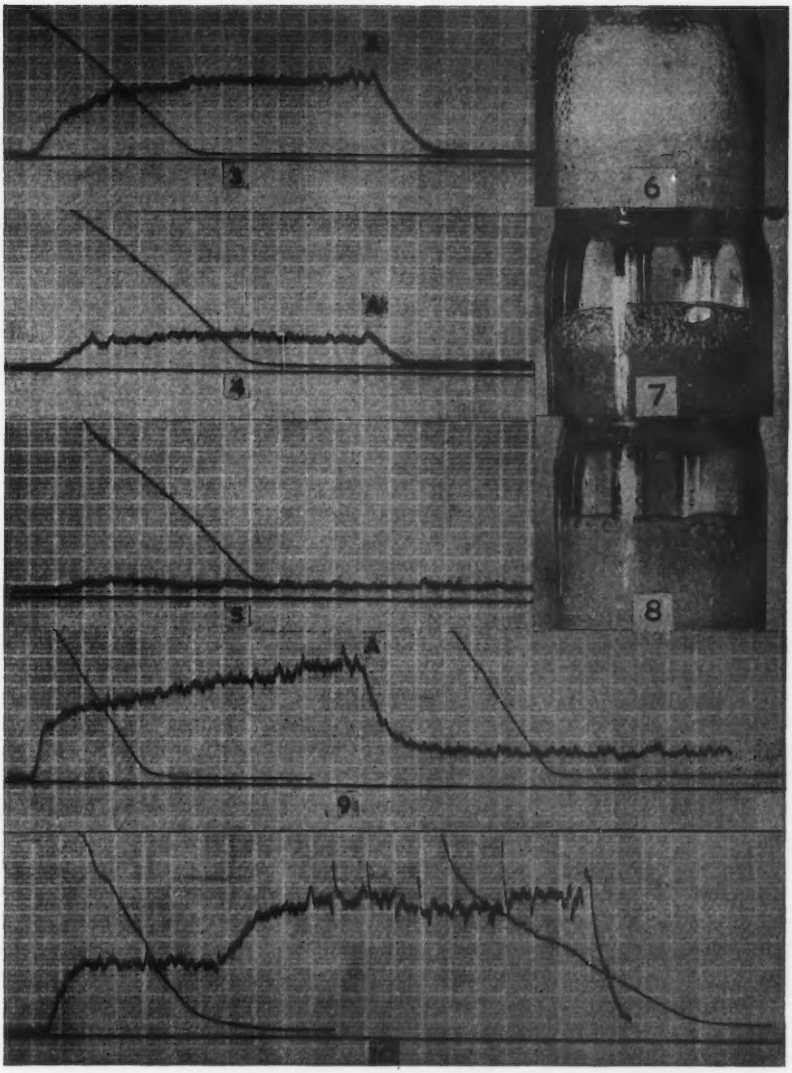
As a result of these findings care was taken to maintain an adequate amount of antifoam in all subsequent work. With this, replicates remained reasonably uniform. The antifoam was introduced on the end of a glass rod through an aperture in the perspex cone.

The Choice of a Substrate

As mentioned above, yeast extract was employed initially in the present experiments. With increased density of cells it was found that the customary 0.2% Difco yeast extract, which was found adequate to maintain a uniform rate of respiration throughout a 40 minute period with the lighter density of cells, was insufficient to meet the new requirements. It was at first suspected that the concentration of the main respiratory component of yeast extract was limiting. However, when 0.2% Difco yeast extract was employed and a continuous record made of the oxygen concentration, the polarogram shown in Fig. 10 was obtained. It was immediately obvious that the change in respiration rate was not merely due to the reduction of substrate concentration below the enzyme saturation level but to a complete change in the type of substrate being used, as indicated by the existence of two "steady state" oxygen levels, one moving rapidly into the other as the first substrate was depleted. This is quite different from the continued upward movement in oxygen concentration which may be expected through simple depletion of a substrate (see Fig. 22).

Rather than increase the concentration of yeast extract to maintain the higher respiration rate, it was decided to test several amino acids as substrates in amounts equal to that of the yeast extract originally employed in an endeavor to ascertain which yielded respiration rates similar to the initial rate on yeast extract. Glycine, alanine, valine, threonine, serine, phenylalanine, glutamic acid, lysine, hydroxy-*l*-proline, arginine, aspartic acid, and leucine were compared with Difco yeast extract and Difco peptone. Similar uptake rates were given by Difco yeast extract, Difco peptone, glutamic acid, and aspartic acid. Alanine was the only other amino acid to yield a high respiration rate which was approximately 80% of that given by yeast extract.

No attempt has been made to analyze the yeast extract during an experiment to determine which of these amino acids, if any, was depleted in the initial stages of the experiment. Peptone was not used experimentally because some component interferes with the polarographic determination of nitrate.



FIGS. 3–8. Apparent steady state oxygen concentrations and rate of foam formation obtained with glutamic acid as the substrate. FIGS. 3 and 6—without antifoam. FIGS. 4 and 7—with small residual antifoam. FIGS. 5 and 8—with adequate antifoam. Antifoam added at point A in FIGS. 3 and 4.

FIG. 9. Progressively increasing oxygen concentration before the addition of antifoam with aspartic acid as substrate.

FIG. 10. Two steady state oxygen levels obtained during the oxidation of 0.2% yeast extract over a 30 minute period.

Glutamic acid was chosen as a substitute for yeast extract because of its ready availability and low cost. It was subsequently discarded in favor of aspartic acid. Reference to Figs. 11–15 will show the reason for this. When the initial and final oxygen uptake rates were determined with glutamic acid in a concentration of 1 mg. per milliliter, the two slopes were found to be nearly identical when pure nitrogen was used in the control experiments (Fig. 11). When air was used at varied gassing rates the respiration rate at the end of the experiment was always considerably greater than at the beginning. There was an obvious adaptation taking place during the experiment. Since this adaptation was absent or very limited when pure nitrogen was used, it was apparent that it was not a simple adaptation to the substrate but one which was to some measure dependent on the presence of oxygen.

This increase in rate of respiration is reflected in a gradually diminishing level of oxygen in the solution (Figs. 14 and 15). The fact that the fall in oxygen concentration approximates linearity with time also indicates that the change is continuous and not abrupt, a fact which was verified by measuring the uptake rate at intervals during the 30 minute period.

The results of some experiments in which glutamic acid was employed are reported in this paper. The fact that steady state levels could not be obtained with the substrate made interpretation of the results difficult.

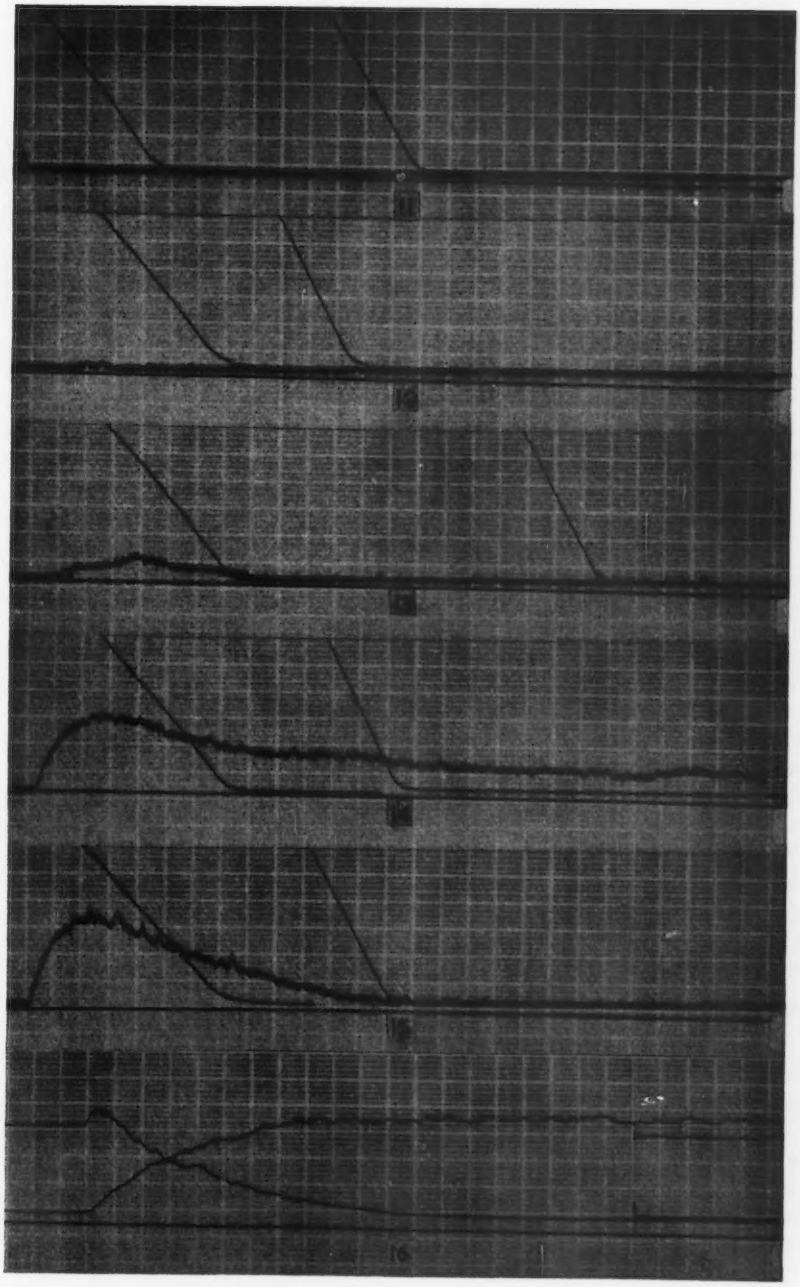
Aspartic acid did not show the same type of adaptation, the initial and final respiration rates being identical provided the substrate was not limiting. It was found that a concentration of 1.5 mg. of aspartic acid per milliliter was necessary for this purpose.

The Relationship of the "Agitated" to "Stationary" Oxygen Concentrations

Vigorous and prolonged gassing of a solution cannot alter the final equilibrium concentration of the dissolved gas. The turbulence of the solution does, however, cause a marked increase in the diffusion current as compared with that of the stationary solution. The relationship between the two values was necessary for the final interpretation of data. Results obtained for a series of oxygen concentrations are illustrated graphically in Fig. 17. The average increase in diffusion current (*Id*) due to turbulence was 33.3%. Turbulence had no detectable effect on the drop size or rate under the conditions in which the electrode was used except for an occasional dislodgment of a partially formed drop. This was apparent from the fact that the number of drops per unit time and the amplitude of the oscillations where the "stationary" and "agitated" *Id* values of different series nearly coincide were the same. The major portion of the current increase is thus due to destruction of the diffusion gradient.

FIGS. 11–15. Initial and final "stationary" oxygen uptake curves and "agitated" oxygen recording over a 30 minute period with glutamic acid (1 mg. per milliliter) as substrate. FIG. 11—pure N_2. FIG. 12—air 25 ml./min. FIG. 13—air 50 ml./min. FIGS. 14 and 15—air 75 ml./min.

FIG. 16. "Gassing out" and oxygen absorption curve obtained with a gas mixture containing 9.5% oxygen.



Bartholemew *et al.* (1) also report that the relationship between the agitated *Id* and oxygen concentration is linear. The magnitude of current increase due to agitation was much greater in the absence of cells.

Plotting of Oxygen Absorption Curves

Oxygen absorption curves were plotted for a number of gas mixtures used at a constant flow rate and for air used at different flow rates. According to Chain *et al.* (3) and Bartholemew *et al.* (1) the rate of oxygen absorption is influenced by cell density.

No attempt has been made to further verify these claims. It has been observed that in the presence of cells there is a reduction in drop size, indicated by a decrease in the amplitude of the oscillation, and a reduction in drop time. The reduction in the $m^{\frac{2}{3}} t^{\frac{1}{6}}$ value, obtained from the weight of mercury collected over a 20 minute period in each case, was 1.0%. Determinations of the oxygen concentration/diffusion current coefficients in the presence and absence of cells showed no significant difference. The oxygen concentration was determined by the ascorbic acid – oxidase method.

Whilst the presence of cells did not introduce any significant change in the coefficient in stationary solutions the magnitude of current increase in the absence of cells in the aerated solutions was greater than that in the presence of cells. For this reason absorption curves were plotted in the presence of cells which had been formalized and washed and showed no respiratory activity over the time interval employed in plotting the curves. The oxygen equivalent of any point on the curve could be obtained from the "agitated" curve in Fig. 17.

One of these curves which is relevant to the discussion is shown in Fig. 16. The "stationary" oxygen concentration was recorded on the paper after 20 minutes' equilibration of the mixture with the gas phase employed. The apparatus leading to the cell was then flushed with pure nitrogen for 10 minutes before the latter was passed through the cell at 100 ml. per minute. When the nitrogen enters initially the turbulence causes the apparent "rise" in oxygen concentration. This is followed by the progressive reduction in oxygen concentration as the nitrogen flows through the mixture at constant speed. The deoxygenation was near completion at 10 minutes. The nitrogen was then replaced by air and the oxygen adsorption curve plotted.

The Effect of Variation in Rate of Air Flow on the Reduction of Nitrate

Three series of experiments were performed in which the air flow rate was fixed at 25, 50, 75, and 100 ml. per minute. Pure nitrogen at 100 ml. per minute was used as the control.

Two of these series were performed with glutamic acid (1 mg. per milliliter) as substrate. All polarograms from one series and the 75 ml. per minute polarogram (Fig. 15) from the second series are shown in Figs. 11–15. The relationship between air flow rate and nitrate reduction for both series are shown in Fig. 18.

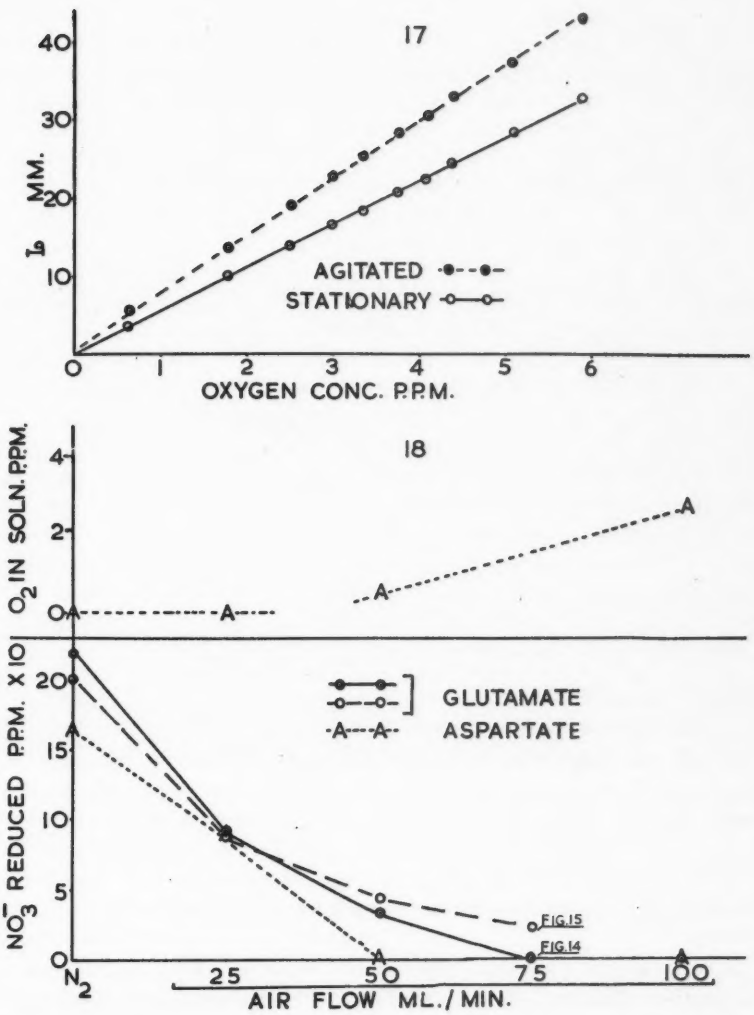


FIG. 17. Relationship between the "agitated" and "stationary" oxygen diffusion currents and oxygen concentration.

FIG. 18. Relationship between nitrate reduction and air flow rate with glutamic acid as substrate and relationship between nitrate reduction, air flow rate, and oxygen in solution with aspartic acid as substrate.

The variation in rate of oxygen uptake as shown by the gradually decreasing oxygen concentration made it impossible to relate the degree of nitrate reduction with the oxygen concentration in solution. It is clear, however, that the amount of nitrate reduced is roughly proportional to the period for which a zero oxygen level is recorded, suggesting that the oxygen must become limiting before the nitrate is reduced. This is best illustrated in the two 75 ml. per minute series in one of which (Fig. 14) the oxygen concentration was always positive and no nitrate was reduced. In the other (Fig. 15) the oxygen concentration was reduced to "zero" during the course of the experiment and a significant amount of nitrate was reduced.

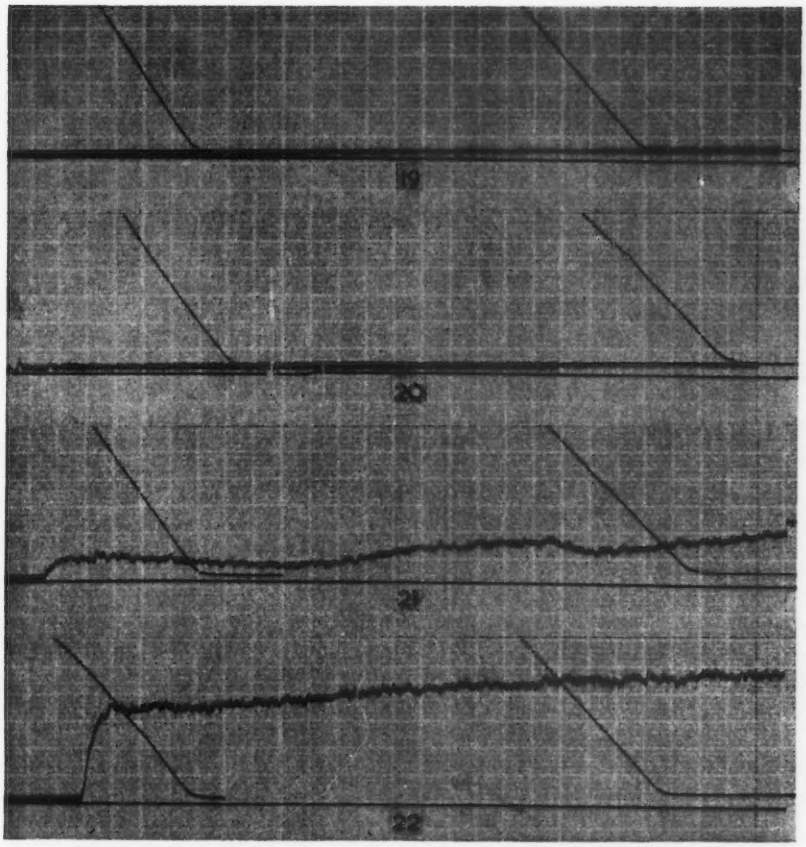
The difference between the two series was probably due to slightly more foaming in one series than the other (*vide supra*).

The third series was made with aspartic acid (1 mg. per milliliter) as the substrate. The polarograms are shown in Figs. 19–22. The concentration of aspartic acid used was not sufficient to maintain enzyme saturation for the full period. The results are quoted because they illustrate the effect of inadequate substrate in such an experiment. The experiment was not repeated since it was obvious on completion that no useful data could be obtained by doing so. That the substrate became limiting is shown by the coupled reduction in the rate of oxygen uptake at the end of the experiment and rise in the oxygen concentration during the experiment. Increase in the concentration of aspartic acid to 1.5 mg. per milliliter satisfied the requirements. The relationship between the rate of air flow to nitrate reduction and oxygen concentration in solution is shown in Fig. 18. It is evident that nitrate is not reduced while the oxygen concentration was equal to that in equilibrium with a gas mixture containing 1.4% oxygen.

Effect of Variation in the Concentration of Oxygen in the Gas Phase at Constant Flow Rate on the Reduction of Nitrate

It was found impracticable to reduce the gas flow rate in small increments from 25 ml. per minute to nil to study the influence of this on nitrate reduction. Apart from the technical difficulties involved there was also the problem of variation in turbulence on the behavior of the electrode. By maintaining a uniform gassing rate at 100 ml. per minute the latter could be standardized. Variation in oxygen concentration could be obtained by varying the gas mixture.

In view of the fact that experiments reported above and in previous publications (Skerman, Lack, and Millis (10), Skerman and MacRae (11)) indicated that no nitrate was reduced while oxygen was detectable in the solution, the approach to the following experiment was to determine the extent of reduction with pure nitrogen and with air and then to continue with a series of gas mixtures with increasing oxygen concentrations up to a level at which nitrate reduction ceased. Three series were performed. In two, owing to time limitations, the experiments were terminated with the gas mixture at

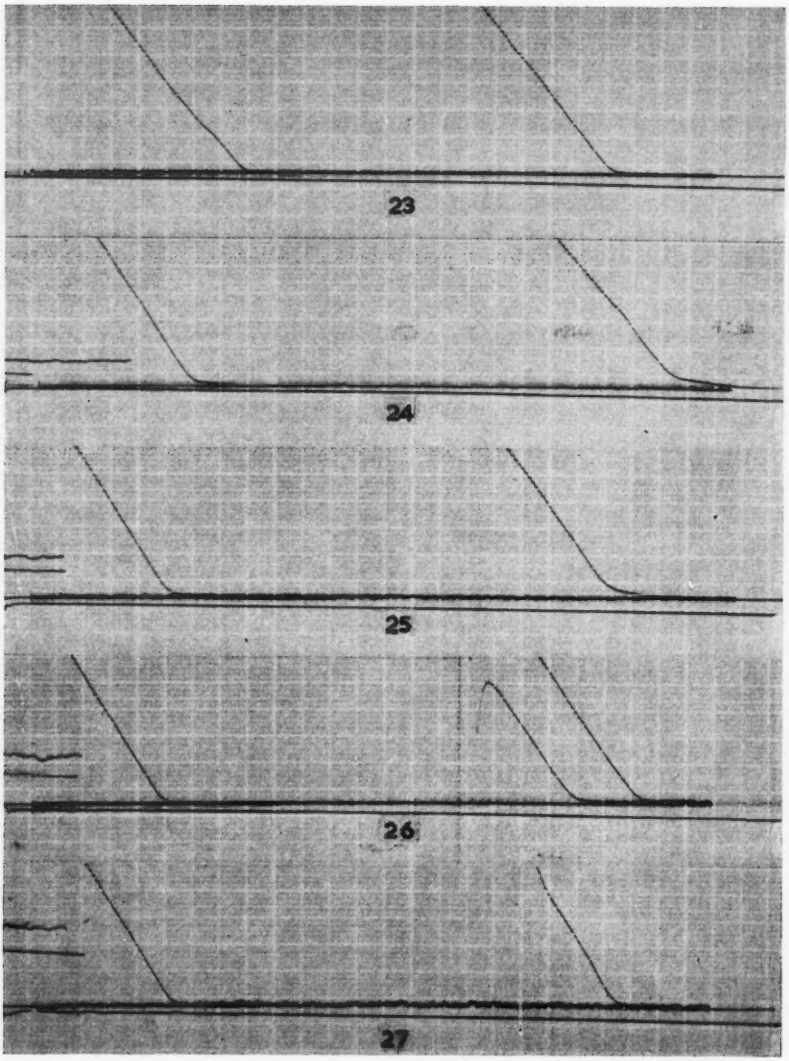


FIGS. 19–22. Initial and final oxygen uptake curves and the "agitated" oxygen level obtained with aspartic acid as substrate (1 mg./ml.). FIG. 19—pure N_2. FIG. 20—air at 25 ml./min. FIG. 21—air at 50 ml./min. FIG. 22—air at 100 ml./min.

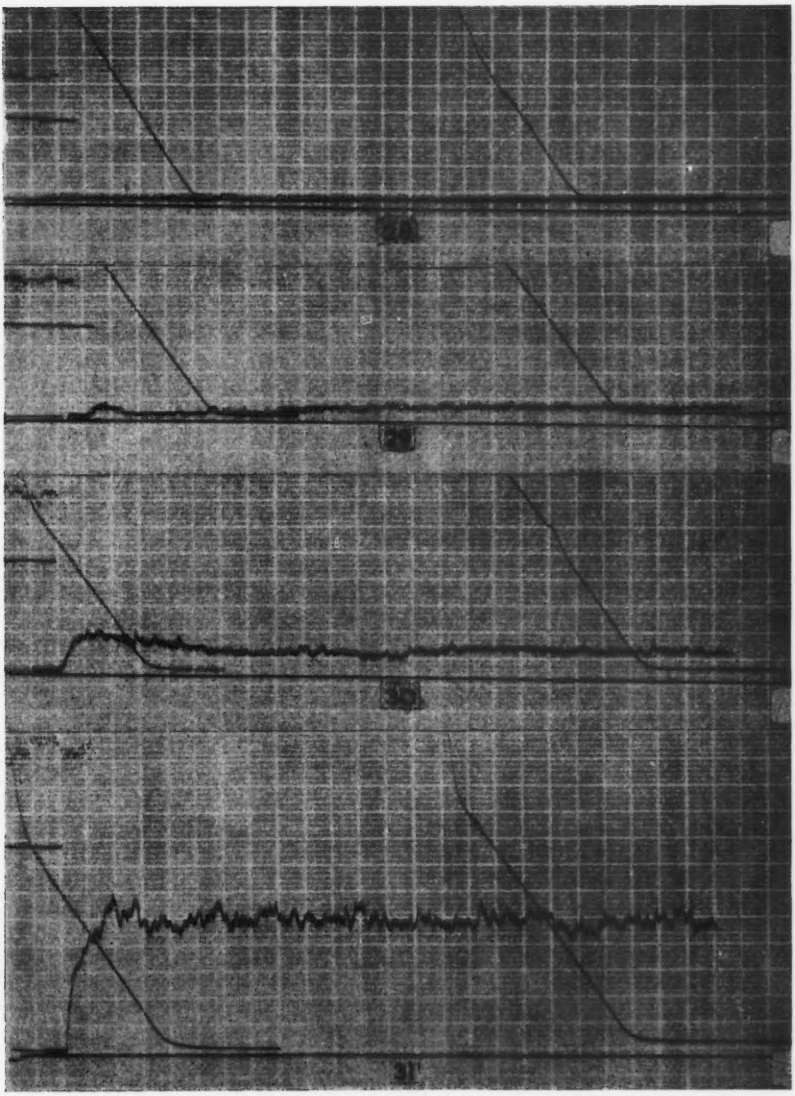
which nitrate reduction no longer occurred. (Each experiment took 1 hour to perform after all materials were prepared.)

In the final series, 10 gas mixtures were used ranging from nitrogen to air, nitrate determinations being made in triplicate at the beginning and end of each experiment. Agreement between replicates was good. These experiments were performed in a continuous series over an 18 hour period.

Representative polarograms from the final series are shown in Figs. 23–31. The relationship between the concentration of oxygen in the gas phase and (*a*) the nitrate reduction and (*b*) the oxygen concentration in the solution are shown in Fig. 33.



FIGS. 23–31. Initial and final oxygen uptake curves and the agitated oxygen level obtained with aspartic acid as substrate. FIG. 23—pure N_2. FIGS. 24–31—increasing concentrations of oxygen equal to 1.25, 2.75, 3.25, 5.5, 8.2, 9.3, 10.75, and 21% in the gas phase respectively. Gas flow rate 100 ml./min./50 ml. solution in each case.



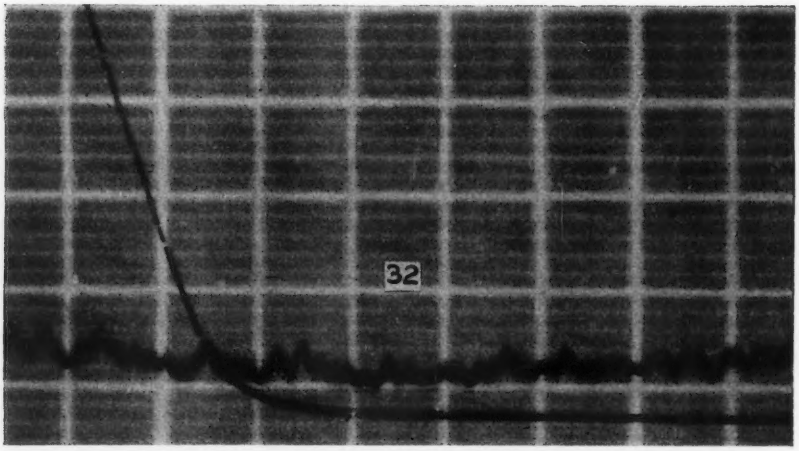


FIG. 32. An enlarged polarogram showing the "agitated" oxygen concentration curve crossing the "stationary" uptake curve in the region of oxygen unsaturation.

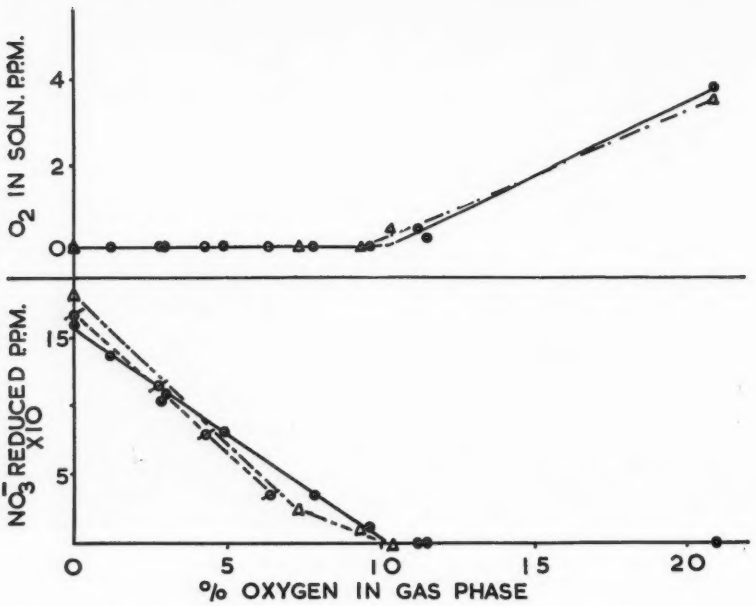


FIG. 33. The relationship between nitrate reduction and (*a*) the oxygen in the gas phase and (*b*) the oxygen in solution using aspartic acid as substrate (1.5 mg./ml.).

Oxygen Steady State Levels and the Curve of Oxygen Uptake by the Organisms

For the purpose of the general discussion Fig. 32 has been inserted. In it both the "stationary" oxygen uptake curve and the "agitated" steady state oxygen level obtained with a gas mixture containing 10.5% oxygen flowing at 100 ml. per minute have been plotted with a galvanometer sensitivity of 1/10 instead of 1/30 as employed with the other polarograms. At this sensitivity, the diffusion current obtained for a mixture saturated with air is too great to be recorded on the paper. Only the end portion of the oxygen uptake curve appears on the polarogram. The figure has been enlarged for purposes of illustration.

Discussion

A number of aspects of the experimental data will be discussed. The principal one—the influence of oxygen on the reduction of nitrate—will be discussed first.

The results obtained with glutamic acid coupled with variation in the flow rate of air (Figs. 11–15) were inconclusive for reasons already stated. The single series in which aspartic acid was used as the substrate with a variation in the flow rate of air (Figs. 19–22) confirmed the previous report that very low oxygen concentrations completely inhibited the reduction of nitrate.

The most informative data were those obtained using aspartic acid coupled with a series of gas mixtures used at constant flow rate of 100 ml. per minute. Fig. 33 shows that the amount of nitrate reduced bears a linear relationship to the concentration of oxygen in the gas phase. However, the same figure shows that for all gas phases at which nitrate reduction occurs the concentration of oxygen in solution remained at zero level, indicating that the rate of solution did not balance the rate of uptake. With the gas phase which finally established a positive level in solution, no nitrate was decomposed. Further aspects of this "end point" will be discussed later.

There are two alternative explanations for the above findings. Either a portion of the population is not getting any oxygen at all and reduces nitrate at a maximal rate while the rest of the population is respiring with oxygen and does not reduce nitrate, or the whole population has the oxygen utilizing enzymes only partially saturated and is simultaneously reducing nitrate. In either case, one may expect that as the amount of oxygen entering solution increases so the amount of nitrate reduced would decrease, the latter ceasing when the available oxygen satisfied the demand. The second alternative is based on the assumption that the rate of electron transfer to oxygen is much greater than to nitrate as stated by Stickland (13). It is, of course, possible that both states coexist in the solution.

An analysis of the experimental procedure provides an answer to a portion, at least, of this question.

Bartholemew *et al.* (1) used the dropping electrode in the turbulent aerated liquid, screening the drop in a stainless steel mesh with a small receptacle at the base to catch the mercury drops. There can be little doubt that no gas

bubbles gained access to the drop during their experiments and that the dropping electrode measured only the oxygen in that portion of the circulating liquid which reached it. In the authors' experiments, a plastic shield was used which did not completely prevent contact between odd bubbles and the mercury drop. It is important to recognize the effect of such contact and distinguish it from an actual rise in the general level of oxygen in solution. When no oxygen is present in the liquid surrounding the drop even the extreme turbulence caused by the movement of 100 ml. of gas per minute through the 50 ml. of solution does not detectably alter the oxygen residual current from that measured in the stationary solution. A bubble which moves in close proximity to the drop or actually comes in contact with it but passes straight by causes a momentary rise in the oxygen concentration at the surface of the drop. This is reflected by a sharp rise and fall in the residual current of the form ___∧___. Small kinks of this nature will be noted in some of the polarograms, the magnitude varying with the magnitude of the current at the time they occur. In contrast to this a general rise in the oxygen level is reflected in a permanent change in the current reading.

The polarized mercury drop may be regarded, in a sense, as a very large bacterial cell. It reacts to oxygen in its vicinity by reducing it as does the bacterial cell. It is sensitive to oxygen concentrations that leave the oxygen reducing enzyme systems of the bacterial cells unsaturated (*vide infra*).

If oxygen were uniformly distributed throughout the solution at a very low concentration this uniform distribution could be expected to hold for all conditions of aeration. Thus with increase in the oxygen concentration in the gas phase there should be a corresponding increase in the oxygen concentration at the electrode surface. The fact that this does not occur with an increase from 0 to 9% oxygen in the gas phase with proportionate increase in the maximum solution rates indicates that the distribution is not uniform, i.e. there exist in the solution areas where no oxygen is present. By the same argument it must be apparent that what oxygen enters the solution must be completely reduced by bacteria before it is capable of reaching the electrode surface by diffusion except in such instances where bubbles move in close proximity to the electrode surface.

The first evidence of oxygen accumulation occurs with *ca.* 10.0% oxygen in the gas phase. Above this level nitrate reduction ceases.

That the plastic shield plays an important role in the experimental results by stagnating part of the liquid may be discounted. In the first place it acts as a baffle which tends to increase rather than decrease turbulence. It exerts no influence on the number of gas bubbles formed at the sintered plate but may cause some dispersion of the gas when the bubbles strike the base of the shield. The only portion of the shield in which liquid could remain quiescent is the small 0.5 ml. cup at the base which holds only 1% of the total volume of liquid in the cell. Quite apart from the obvious stirring caused by the movement of liquid and bubbles through the holes in the shield, it is being continuously

stirred by falling mercury drops and reduced in volume as the mercury accumulates. In addition the gas adsorption curves obtained fit the equations given for such curves in open solutions.

Whilst the evidence indicates that a portion of the population is not getting any oxygen until the oxygen solution rate exceeds the uptake rate, it is not to be supposed that these cells which do obtain oxygen are capable of respiring at maximal rate. It has been shown by several workers that above a critical oxygen level the oxygen uptake curve is one of zero order. Below this critical level it changes to one of first order (Fig. 32). It is possible that some organisms closely attached to the bubble and adhering to it during its transit through the solution may be able to respire at maximal rate. As the distance of the bacterial cell from the bubble increases the degree of saturation will decrease so that a portion of the population will inevitably respire at a submaximal rate and for a very limited period. Whether cells whose oxygen utilizing enzymes are only partially saturated are able to reduce nitrate remains to be answered.

If, as the evidence indicates, the nitrate reduction ceases as soon as the oxygen utilizing enzymes are saturated, it should be possible to demonstrate that reduction ceases when the maximum solution rate of the oxygen just balances the maximum oxygen uptake rate by the cells.

The equation governing gas absorption and steady state levels at which the rate of solution balances the rate of oxygen uptake have been discussed by Wise (14), Hixson and Gaden (5), Bartholemew *et al.* (1), and reviewed critically by Finn (4).

In the derivation of the steady state equation these authors have been concerned with levels of oxygen greater than the critical concentration, i.e. where the oxygen uptake curve is of zero order. The steady state equation covering this obviously does not apply to the subcritical level where the rate of oxygen uptake varies with the oxygen concentration. It is with this latter portion of the curve that one has to deal first as the oxygen concentration is gradually increased from zero to the critical level.

Fig. 29 is an example of a case where the steady state level of oxygen crosses the oxygen uptake curve in the region in which the oxygen utilizing enzymes are not saturated. Care must be taken in interpreting the polarogram. The oxygen uptake curves have been plotted in the stationary solution while the steady state recording is for the turbulent solution. The magnitude of *Id* in the latter is 33.3% greater for an equal oxygen concentration than the former (see Fig. 17) and has to be reduced accordingly. This relationship is better illustrated in Fig. 32 where an essentially similar state has been plotted at a higher galvanometer sensitivity.

Fig. 16 shows the oxygen absorption curve plotted with an almost identical gas mixture with a suspension of non-respiring cells. The curve fits the equation

[1] $$dC/dt = k(Ce - C),$$

where Ce = concentration of oxygen in equilibrium with the gas phase,
C = concentration in solution at any time interval t,
k = absorption constant.

The equation, on integration, may be expressed as

[2] $$k = (2.303/t) \log_{10} Ce - (2.303/t) \log_{10}(Ce - C)$$

or

[3] $$k = (2.303/t) \log_{10}[Ce/(Ce - C)].$$

Whether Ce and C are expressed as mM./liter, g-mol./ml., or galvanometer deflection (polarographic Id) does not matter. The answer in each case will be the same.

From equation [2] if t is plotted against $\log_{10}(Ce - C)$ the result should be a straight line from the slope of which k may be derived. The slope may be obtained either by dividing the difference between two $\log_{10}(Ce - C)$ values by the corresponding time interval or by measuring the angle subtended by the slope with the ordinate and taking its tangent. If the same numerical value is to be obtained from both methods it is essential that the ordinate and abscissa be in the same units (i.e. in millimeters or inches, etc.). For example in Fig. 16, Ce = 22.5 mm.; hence

$$\text{when } C = 0, \quad \log_{10}(Ce - C) = 1.3522 \text{ at } t = 0 \text{ (in mm.)},$$
$$\text{and when } C = 19.5, \log_{10}(Ce - C) = 0.5119 \text{ at } t = 43 \text{ (in mm.)},$$
$$\text{and the slope} = (1.3522 - 0.5119)/43 = 0.0196.$$

When $\log_{10}(Ce - C)$ is plotted on a centimeter scale and t on a millimeter scale the angle subtended by the slope on the ordinate = 11°, and tan 11° = 0.1944. Dividing this value by 10 to reduce the abscissa scale to millimeters gives tan θ = 0.0194.

From these values,

$$k = 2.303 \times \text{slope}^*,$$
$$= 2.303 \times 0.0194 = 0.045.$$

By substituting values for Ce, C, and t (all expressed in millimeters) in Equation [3], the following values for k were obtained: 0.0494, 0.0472, 0.0463, 0.044, 0.0415, 0.0415, 0.0446, 0.0453. The average of these values is 0.045.

In the example cited the time scale is in millimeters and k may therefore be expressed as 0.045 per mm. In the time scale, 16.5 mm. = 1 minute, $k = 0.045 \times 16.5$ per minute. This is equivalent to the ϕ L of Wise (14). If multiplied by 60, $k = K_L a$ (Finn (4)) = 44.5 per hour.

From Fig. 16 the maximum solution rate = $K_L a\ Ce$, where $Ce = 17 \times 0.25/38.5$ mM./liter, and thus

$K_L a\ Ce = 44.5 \times 17 \times 0.25/38.5$ mM./liter/hour = 4.95 mM./liter/hour.

(Note: the 17 is derived from the "stationary" saturated level in Fig. 16 and the 38.5 is the equivalent reading for a similar solution saturated with air. The 0.25 mM./liter is only approximate.)

The slope of maximum oxygen uptake in Fig. 32 is 1.428 (= tan 55°). If this is adjusted to that theoretically occurring in the agitated solution using data from Fig. 17 the slope becomes 1.8807 (= tan 62°).

*Chain *et al.* (3) incorrectly state this as 2.303/slope.

Substituting the value 0.045 per mm. for k and 22.5 mm. for Ce in the equation

$$dC/dt = k(Ce - C),$$

the maximum solution rate when $C = 0$,

$$dC/dt = 0.045 \times 22.5;$$

then $\tan \theta = 1.0125$,

and $\theta = 45.5°$.

Since this is considerably less than the slope of uptake (62°) the oxygen concentration cannot reach the level at which the zero order reaction occurs. If the angle (45.5°) is reduced to conform to the stationary readings, it becomes 38°. A tangent meeting the abscissa at an angle of 38° in Fig. 32 touches the first order portion of the uptake curve at a point 2.25 mm. (original scale) above the zero. This value—increased again by 33.3% to conform to the agitated state (=3.0)—indicates the steady state level to which the oxygen may be expected to rise. This coincides very closely with the observed curve. At the galvanometer sensitivity of 1/30 used in Fig. 29 this represents a rise of 1 mm., which is approximately the observed value.

It is obvious from Fig. 29 that while a minor portion of the population in close proximity to the ascending bubbles may momentarily be fully satisfied, the amount of oxygen entering the solution is sufficient to meet only partial requirements of the whole bacterial population. At this level the amount of nitrate reduced fell within the limits of experimental error and may not be significant. Any higher concentration may therefore be regarded as completely inhibitory.

The observations indicate that a further solution of this problem requires a study of the influence of oxygen concentrations in solution in equilibrium with oxygen concentrations between 0 and 1% in the gas phase. This is the range of concentrations covering the first order portion of the uptake curve. It may be possible to produce such apparent concentrations by varying the concentration of oxygen in the gas phase within fine limits and applying very rigid control over the gas flow rate. Resolution of the differences would require an increased sensitivity of the instrument. Increase in instrument sensitivity means an increase in magnitude of all current measurements including the fluctuations. On the average these fluctuations are equivalent to 2 to 2.5 mm. at low oxygen concentrations (see Fig. 32), i.e. approximately half the value of the oxygen concentrations covering the first order curve. Moss (8) does not indicate variation with his instrument but variation of a similar order to that in Fig. 31 is shown by Chain *et al.* (3 (Fig. 6, p. 24)).

Quite apart from the difficulty of measuring the oxygen concentration accurately there is the question of interpretation. Any method using gas mixtures to establish the equilibrium must fail to give a true picture of the inhibition because it is impossible to separate those components of the population which are saturated from those only partially saturated, the electrode measuring essentially the net result.

The only other means of maintaining a level of oxygen would be by mixing in with the suspension a fluid saturated with oxygen. Besides being subject

to objections similar to those applying to the use of gas mixtures this would introduce a dilution effect to render interpretation still more complex.

Any attempt to work within the available oxygen in solution would be impossible. To do so would necessitate a reduction in population density of such an order that detection of nitrate reduction would hardly be practicable. Apart from this a steady level of oxygen concentration could not be maintained.

More useful information is likely to come from a study of the relative rates of respiration with oxygen and nitrate in relation to the amount of substrate oxidized.

A second aspect of the results is the effect of foam on the oxygen concentration in the solution as shown by Figs. 3–8. Chain and Gualandi (3) using the vortex method of stirring showed quite clearly that the addition of antifoam caused a sharp increase in the rate of solution of oxygen and hence on ultimate steady state levels. He attributed this to the destruction of foam which was mechanically retained in solution and gradually depleted of oxygen. Starks and Koffler (12) reported that the formation of a foam on the surface of liquid in a rotating flask retards the rate of solution of oxygen. This is due, no doubt, to the blanketing effect of the foam, which prevents the entry of fresh air and at the same time results in the depletion of oxygen in the lower layers of the foam.

The conditions existing in experiments reported in this paper are quite different from the above. Continual introduction of fresh gas is occurring at the base of the container so that the lower layer of bubbles in the surface foam is continually renewed. The contribution of oxygen to the bulk of the solution by the breaking of the surface layers of the foam is remarkable. The rapid rise in the diffusion current where no antifoam is added is clear evidence of the rapidity with which this oxygen is redistributed through the solution since the dropping electrode is mounted *ca.* 45 mm. below the surface.

The contribution made by the foam to the oxygen concentration in the solution probably explains why it was possible in the previous paper (Skerman and MacRae (11)) to maintain a positive oxygen balance with 1.5% oxygen in the gas phase whilst it was not possible to do this with 9.0% in the defoamed solution. It also may explain why it proved difficult to obtain maximal nitrate reduction when nitrogen passed through a copper reducing column was used to break the foam. It is conceivable that a small amount of oxygen was present in this gas or became mixed with it above the liquid level.

The demonstration (Fig. 10) that variation in the initial and final uptake rates with yeast extract was due to a complete change in the substrate being used was quite unexpected. The observation suggests a useful tool for studying the effect of supplying organisms with two or more oxidizable substrates simultaneously.

The observation of the steady state level of oxygen concentration using single substrates has proved useful in determining the amount of substrate required for any length of experiment. It merely requires that the density

of cells to be employed be mixed with the chosen quantity of substrate in the cell and the air flow rate adjusted so that the steady state level is approximately half the saturation level. A continuous recording can then be made until the level shows an abrupt change due to desaturation of the enzymes. This method has been employed in choosing substrate concentrations for the study of the effect of oxygen on nitrite decomposition.

Implications of the Results on the Decomposition of Nitrate in the Soil

There have been numerous reports of extensive decomposition of nitrate occurring in apparently well-aerated soils. The most dramatic example of this is possibly that provided by Broadbent (2).

The authors' results suggest that where nitrate reduction occurs in soils there is a depletion of oxygen in the soil solution. Such a state is readily understandable. Maximal microbial activity occurs when soils are 60–70% saturated with water. A portion of the soil water is retained as an exposed film over the soil particles and a portion retained in capillaries between particles. Convectional movement in fluid retained by capillarity is very poor and the solution of oxygen is reduced to that of diffusion and thermal convection across relatively narrow surfaces. Whether the solution rate meets the demand of the bacterial population will depend on all factors determining the rate of respiration, the principal factors being the types of organisms, availability of readily oxidized substrate, and temperature. Evolution of carbon dioxide will also tend to sweep oxygen out of the soil solution. In capillary held water the likelihood of anoxybiontic conditions existing is high whereas in the exposed surface film a satisfactory oxygen supply is quite possible.

In soils where the available readily oxidizable organic matter is low it is possible that respiration will increase as the soil moisture decreases with a concurrent increase in the concentration of solutes. The residual moisture would be maintained mainly in capillary form. Nitrate reduction in relatively dry soil is therefore to be expected.

For similar reasons water logging per se need not result in denitrification. This will depend on the availability of oxidizable substrate and oxygen in the soil solution.

Acknowledgments

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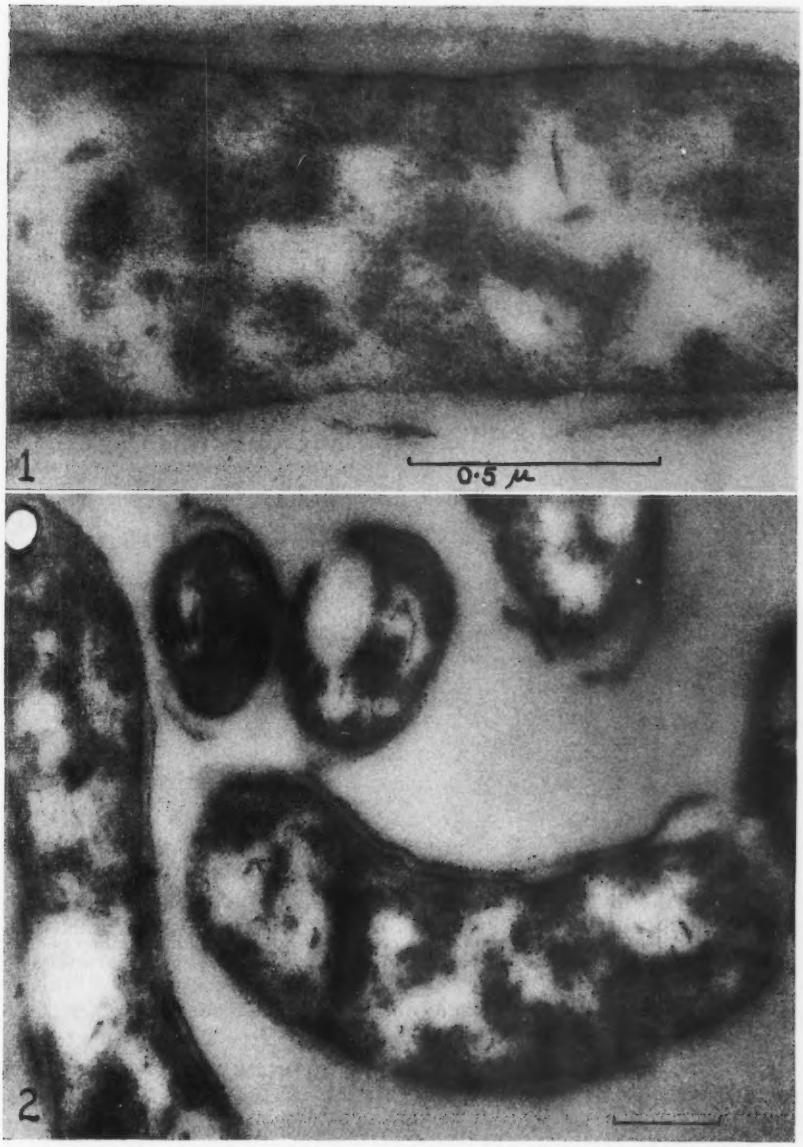

FIGS. 1 and 2. Micrographs of sections of a *Spirillum* sp. showing a disintegrating and partially lost cell wall and a dense membrane in close apposition to the protoplast, which we believe to be cytoplasmic membrane. The discontinuities in the protoplast are due to damage during polymerization of the embedding medium. (The markers approximate 0.5 μ in length in all the figures.)

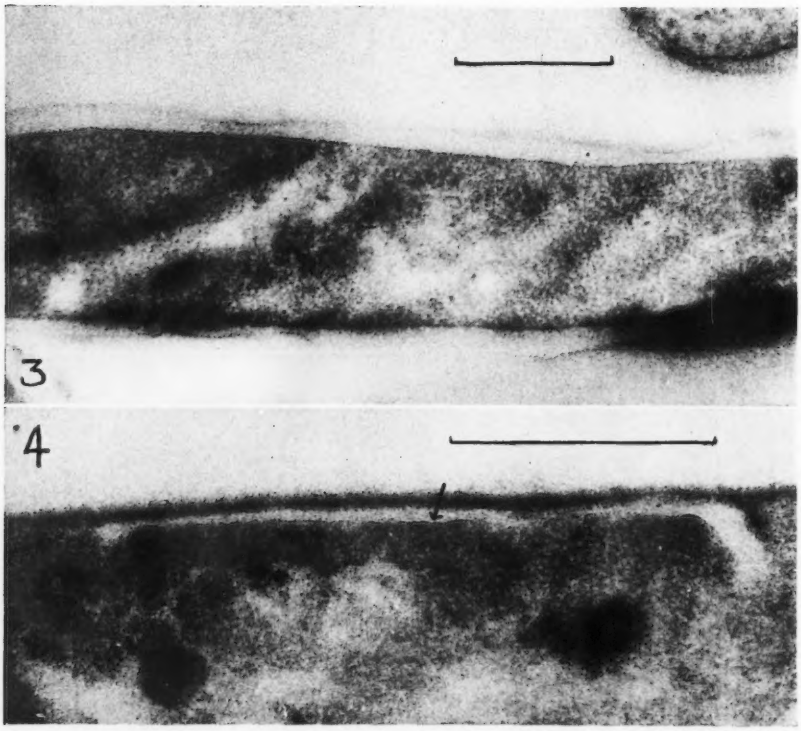

FIG. 3. From the same embedding as Figs. 1 and 2 but showing better preservation of the protoplast.

FIG. 4. A portion of a section of *Bacillus cereus* showing a slight separation of the thick cell wall from the protoplast. At the surface of the protoplast there is a very delicate membranous structure (arrow). The apparent circular inclusion at the lower left is a contamination of the specimen.

NOTES

DIRECT EVIDENCE FOR A CYTOPLASMIC MEMBRANE IN SECTIONED BACTERIA

R. G. E. MURRAY

The evidence for a cytoplasmic membrane at the surface of the protoplast of bacteria is largely indirect. Plasmolysis of a number of larger bacteria indicates the possibility of an osmotic barrier separate from the cell wall. Methods for staining the cell surfaces have been shown to stain the cell wall lightly or not at all, while staining intensely a very thin layer at the surface of the protoplast. These cytological methods and an appraisal of the evidence up to 1953 have been presented in a previous paper from this laboratory (4). The ability of many bacteria to withstand and grow in a wide variety of ionic and osmotic environments argues for a physiologically active superficial layer. In fact, energy is required for the exclusion of salts from the protoplast of a halophilic organism (5). A selective metabolic barrier, able to operate against very considerable diffusion pressures, would be expected to be a definite structure. Indeed, an estimate of probable thickness has been made (3) and is of the order of 100 Å.

Electron micrographs of partially lyzed bacteria have only occasionally been suggestive (see (2) and (4)). The best direct evidence of a cytoplasmic membrane has been provided by the elegant work of Weibull (7). He digested the cell walls from *Bacillus megaterium* with lysozyme and obtained spherical protoplasts which retain the permeability properties and many of the capabilities of the intact cell. These can be lyzed, leaving behind a 'ghost' of membrane-like appearance, which may well be a cytoplasmic membrane.

Hopes that the ultrathin sectioning techniques would be definitive have been almost consistently disappointing, as Bradfield (2) has pointed out. Tomlin and May (6) noted "a very obvious plasma membrane" in a section of one *Escherichia coli* cell and stated that this structure was "a little thinner than" 100 Å. Birch-Andersen, Maaløe, and Sjöstrand (1) did not differentiate between cell wall and cytoplasmic membrane; yet one of their sections of *E. coli* (their Fig. 1) shows what might be interpreted, nowadays, as a cytoplasmic membrane on the surface of the protoplast and distinct from the double contoured cell wall. Other workers have, to our knowledge, either noted the absence of an apparent cytoplasmic membrane or made no mention of the structure. In all cases, the preparations have been made by comparable techniques: fixation with osmium tetroxide, dehydration with alcohol, and embedding in methacrylate (usually *n*-butyl methacrylate mixed with varying proportions of methyl methacrylate).

Our experience is that we have seen no sign of a cytoplasmic membrane in micrographs of sections from virtually all embeddings. However, in one of three embeddings of a species of *Spirillum*, isolated from river water, there was a clearly defined and strongly scattering membrane always closely applied to the surface of the cytoplasm (Figs. 1–3). This was distinct and separate from the cell wall, which was partially disintegrated as well as separated from the protoplast owing to a combination of shrinkage during dehydration, polymerization damage, and solution during treatments. We may postulate that it represents a true cytoplasmic membrane because of its location and the apparent separation from cell wall. Its thickness approximates 60 Å.

We have not been so fortunate with sections of other kinds of bacteria. One embedding of *Bacillus cereus* (out of more than 100) shows a similar but very thin (25–50 Å) membrane of similar behavior (Fig. 4). Smaller numbers of embeddings of *E. coli*, *Caryophanon latum*, and various species of *Corynebacterium*, *Nocardia*, and *Micrococcus* have all failed to show such a structure. We cannot guess what conditions may be required for the preservation of this physiologically important region of the cell. It is obvious that present methods are not good enough. Yet these accidental findings lend credence to a physiologically and structurally differentiated protoplasmic surface and give hope for more informative demonstrations in the future.

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